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Figure Legends

Figure 1: Plasma nitrite concentration ($[NO_2^-]$) following 4-6 days of dietary nitrate or placebo supplementation. The upper panel shows the group mean (± SEM) values of plasma NO_2^- on days 4, 5 and 6 of supplementation with either nitrate (black circles) or placebo (grey squares). The blood samples for $[NO_2^-]$ determination were taken before each of the six exercise bouts that were completed in each condition (bouts 1, 2, 3 and 5 were moderate, and bouts 4 and 6 were severe; see text for further details). Note the significantly greater plasma $[NO_2^-]$ following dietary nitrate supplementation: # and * denote significant difference from placebo at corresponding time point at the 5% and 1% levels of significance, respectively. The lower panel shows the individual (dashed grey lines) and mean ± SEM (solid black line) values for plasma $[NO_2^-]$ measured over days 4, 5 and 6.

Figure 2: Systolic blood pressure (SBP) following 4-6 days of dietary nitrate or placebo supplementation. The upper panel shows the group mean (± SEM) values of SBP on days 4, 5 and 6 of supplementation with either nitrate (black circles) or placebo (grey squares). Note the significantly lower SBP following dietary nitrate supplementation: # and * denote significant difference from placebo at corresponding time point at the 5% and 1% levels of significance, respectively. The lower panel shows the individual (dashed grey lines) and mean ± SEM (solid black line) values for SBP measured over days 4, 5 and 6.

Figure 3: Group mean changes in the parameters of muscle oxygenation following nitrate and placebo supplementation before and during a step increment to a moderate-intensity cycle work rate. Responses following nitrate supplementation are shown as filled circles, while the placebo responses are shown as open circles. The dashed vertical line represents the abrupt imposition of a moderate work rate from a baseline of 'unloaded' cycling. Panel A: [HHb]; Panel B: $[O_2Hb]$; Panel C: [Hbtot]. For each individual, the responses to four like-transitions were averaged together prior to analysis. Note the reduction in the amplitude of the [HHb] response and the greater $[HbO_2]$ and [Hbtot] before and during moderate exercise following dietary nitrate supplementation. Error bars not shown for clarity but are see Table 1 for further details.

Figure 4: Group mean changes in the parameters of muscle oxygenation following nitrate and placebo supplementation before and during a step increment to a severe-intensity cycle work rate. Responses following nitrate supplementation are shown as filled circles, while the placebo responses are shown as open circles. The dashed vertical line represents the abrupt

939 imposition of a severe work rate from a baseline of 'unloaded' cycling. Panel A: [HHb]; Panel
940 B: [O_2Hb]; Panel C: [Hbtot]. For each individual, the responses to two like-transitions were
941 averaged together prior to analysis. The NIRS-derived parameters were not appreciably
942 different before or during severe exercise following dietary nitrate supplementation. Error bars
943 not shown for clarity but are see Table 1 for further details.
944
945 **Figure 5:** Pulmonary oxygen uptake ($\dot{V}o_2$) response following nitrate and placebo
946 supplementation during a step increment to a moderate-intensity work rate. Responses
947 following nitrate supplementation are shown as filled circles, while the placebo responses are
948 shown as open circles. The dashed vertical line represents the abrupt imposition of the
949 moderate work rate from a baseline of 'unloaded' cycling. The upper panel shows the $\dot{V}o_2$
950 response of a representative individual (data are shown at 5 s intervals). The middle panel
951 shows group mean $\dot{V}o_2$ response with error bars shown every 30 s for clarity. The oxygen cost
952 of moderate exercise was significantly reduced following beetroot supplementation. The lower
953 panel shows individual changes in the amplitude of the $\dot{V}o_2$ response to moderate exercise
954 following nitrate supplementation (dashed grey lines) along with the group mean change (solid
955 black line). For each individual, the responses to four like-transitions were averaged together
956 prior to analysis. Note that the effect was observed in all participants.
957
958 **Figure 6**: Pulmonary oxygen uptake ($\dot{V}o_2$) response following nitrate and placebo
959 supplementation during a step increment to a severe-intensity work rate. Responses following
960 nitrate supplementation are shown as filled circles, while the placebo responses are shown as
961 open circles. The dashed vertical line represents the abrupt imposition of the severe work rate
962 from a baseline of 'unloaded' cycling. The upper panel shows the $\dot{V}o_2$ response of a
963 representative individual (data are shown at 5 s intervals). The data are plotted as a fraction of
964 the $\dot{V}o_2$ fundamental component amplitude to more clearly illustrate the slower phase II $\dot{V}o_2$
965 kinetics and reduced $\dot{V}o_2$ slow component following nitrate supplementation. The middle panel
966 shows group mean $\dot{V}o_2$ response with error bars shown every 30 s for clarity. The group mean
967 ± SEM $\dot{V}o_2$ at task failure is also shown. The lower panel shows individual changes in the
968 tolerance of severe exercise following nitrate supplementation (dashed grey lines) along with
969 the group mean change (solid black line).
970
971
972
973

974 **Table 1. Mean ± SD NIRS-derived deoxygenated haemoglobin (HHb), oxygenated**
975 **haemoglobin (O_2Hb) and total haemoglobin (Hbtot) dynamics during moderate- and**
976 **severe-intensity exercise following supplementation with nitrate and placebo.**
977

	Placebo	Nitrate
Moderate-intensity exercise		
[HHb]		
[HHb] Baseline (AU)	-132 ± 84	-131 ± 96
[HHb] 120-s (AU)	-41 ± 56	-54 ± 74
[HHb] End (AU)	-51 ± 55	-55 ± 83
[HHb] Mean Response Time (s)	32 ± 8	29 ± 9
[HHb] Amplitude (AU)	88 ± 38	78 ± 34#
[O_2Hb]		
[O_2Hb] Baseline (AU)	-29 ± 74	21 ± 51#
[O_2Hb] 120-s (AU)	-80 ± 72	-15 ± 30#
[O_2Hb] End (AU)	-5 ± 67	25 ± 39
[Hbtot]		
[Hbtot] Baseline (AU)	-160 ± 129	-110 ± 89#
[Hbtot] 120-s (AU)	-47 ± 89	-29 ± 70
[Hbtot] End (AU)	-57 ± 92	-30 ± 81
Severe-intensity exercise		
[HHb]		
[HHb] Baseline (AU)	-142 ± 95	-104 ± 89
[HHb] 120-s (AU)	176 ± 107	202 ± 125
[HHb] End (AU)	215 ± 110	246 ± 126
[HHb] Primary time constant (s)	9 ± 2	11 ± 2
[HHb] Primary time delay (s)	8 ± 1	8 ± 2
[HHb] Primary amplitude (AU)	300 ± 70	287 ± 103
[HHb] Slow phase amplitude (AU)	63 ± 27	67 ± 16
[O_2Hb]		
[O_2Hb] Baseline (AU)	96 ± 119	57 ± 91
[O_2Hb] 120-s (AU)	-147 ± 66	-176 ± 68
[O_2Hb] End (AU)	-133 ± 59	-166 ± 65
[Hbtot]		
[Hbtot] Baseline (AU)	-46 ± 116	-47 ± 69
[Hbtot] 120-s (AU)	29 ± 121	26 ± 105
[Hbtot] End (AU)	82 ± 110	80 ± 104

978
979 # = significantly different from placebo (P<0.05).
980
981
982
983
984

985 **Table 2. Mean ± SD ventilatory and gas exchange dynamics during moderate- and**
986 **severe-intensity exercise following supplementation with nitrate and placebo.**
987

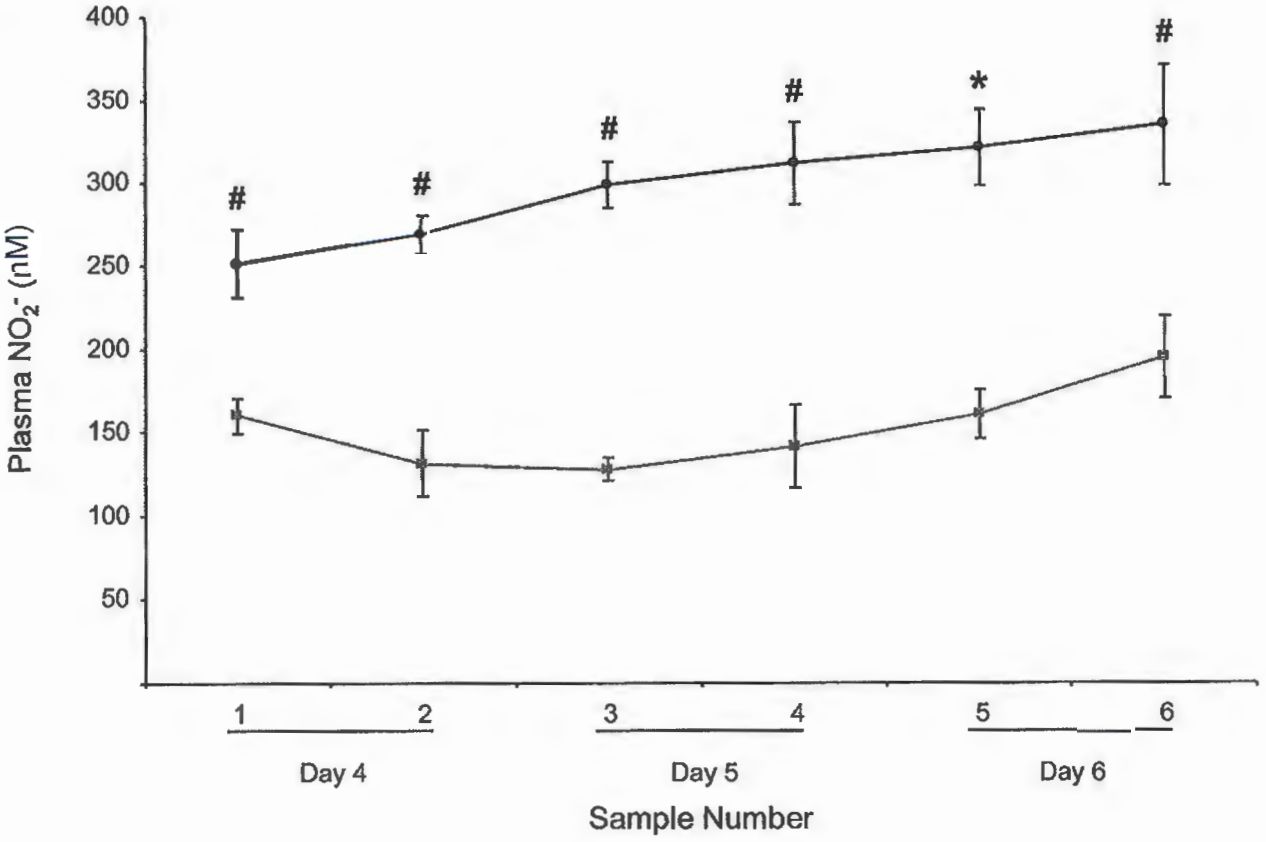
	Placebo	Nitrate
Moderate-intensity exercise		
Oxygen Uptake ($\dot{V}O_2$)		
Baseline ($L \cdot min^{-1}$)	0.91 ± 0.09	0.93 ± 0.05
End-exercise ($L \cdot min^{-1}$)	1.52 ± 0.12	1.45 ± 0.13*
Phase II Time Constant (s)	26 ± 7	29 ± 6
Mean Response Time (s)	39 ± 8	45 ± 4
Primary Amplitude ($L \cdot min^{-1}$)	0.64 ± 0.15	0.52 ± 0.15*
Primary Gain ($ml \cdot min^{-1} \cdot W^{-1}$)	10.8 ± 1.6	8.6 ± 0.7#
Expired Carbon Dioxide ($\dot{V}CO_2$)		
Baseline ($L \cdot min^{-1}$)	0.85 ± 0.08	0.84 ± 0.05
End-exercise ($L \cdot min^{-1}$)	1.31 ± 0.15	1.32 ± 0.15
Minute Ventilation ($\dot{V}_E$)		
Baseline ($L \cdot min^{-1}$)	25 ± 2	24 ± 1
End-exercise ($L \cdot min^{-1}$)	36 ± 4	34 ± 3
Respiratory Exchange Ratio		
Baseline	0.93 ± 0.07	0.90 ± 0.07
End-exercise	0.90 ± 0.05	0.91 ± 0.02
Severe-intensity exercise		
Oxygen Uptake ($\dot{V}O_2$)		
Baseline ($L \cdot min^{-1}$)	0.99 ± 0.10	0.96 ± 0.07
End-exercise ($L \cdot min^{-1}$)	3.87 ± 0.29	3.82 ± 0.28
Phase II Time Constant (s)	33 ± 11	40 ± 13#
Primary Amplitude ($L \cdot min^{-1}$)	2.19 ± 0.17	2.35 ± 0.18#
Primary Gain ($ml \cdot min^{-1} \cdot W^{-1}$)	9.0 ± 0.7	9.4 ± 0.6
Slow phase amplitude ($L \cdot min^{-1}$)	0.74 ± 0.24	0.57 ± 0.20#
Slow component amplitude (%)	25 ± 6	19 ± 6#
Overall Gain ($ml \cdot min^{-1} \cdot W^{-1}$)	11.6 ± 0.9	10.8 ± 0.8*
Overall Mean Response Time (s)	75 ± 16	71 ± 16
Expired Carbon Dioxide ($\dot{V}CO_2$)		
Baseline ($L \cdot min^{-1}$)	0.85 ± 0.23	0.86 ± 0.10
End-exercise ($L \cdot min^{-1}$)	3.99 ± 0.32	4.03 ± 0.43
Minute Ventilation ($\dot{V}_E$)		
Baseline ($L \cdot min^{-1}$)	24 ± 7	25 ± 3
End-exercise ($L \cdot min^{-1}$)	140 ± 14	139 ± 21
Respiratory Exchange Ratio		
Baseline	0.86 ± 0.17	0.89 ± 0.09
End-exercise	1.04 ± 0.05	1.05 ± 0.05

988
989 # = significantly different from placebo ($P<0.05$); * = significantly different from placebo
990 ($P<0.01$).
991

992
993 **Table 3. Mean ± SD heart rate and blood lactate responses to moderate- and severe-**
994 **intensity exercise following supplementation with nitrate and placebo.**
995
996

	Placebo	Nitrate
Moderate-intensity exercise		
Heart Rate		
Baseline ($b \cdot min^{-1}$)	81 ± 9	81 ± 8
End ($b \cdot min^{-1}$)	98 ± 12	98 ± 13
Time Constant (s)	28 ± 12	31 ± 16
Amplitude ($b \cdot min^{-1}$)	17 ± 6	16 ± 7
Blood [Lactate]		
Baseline (mM)	1.0 ± 0.5	0.9 ± 0.3
End (mM)	1.2 ± 0.7	1.1 ± 0.2
Δ (mM)	0.2 ± 0.2	0.2 ± 0.2
Severe-intensity exercise		
Heart Rate		
Baseline ($b \cdot min^{-1}$)	85 ± 8	88 ± 8
End ($b \cdot min^{-1}$)	170 ± 8	170 ± 8
Time Constant (s)	16 ± 10	17 ± 5
Blood [Lactate]		
Baseline (mM)	1.0 ± 0.2	1.1 ± 0.5
End (mM)	6.9 ± 1.6	6.9 ± 1.2
Δ (mM)	5.9 ± 1.6	5.7 ± 1.0
Exhaustion (mM)	10.0 ± 1.9	10.0 ± 1.7

997


400
350
300
250
200
150
100
50
Plasma NO_2^- (nM)
#
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*
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1
2
3
4
5
6
Day 4
Day 5
Day 6
Sample Number

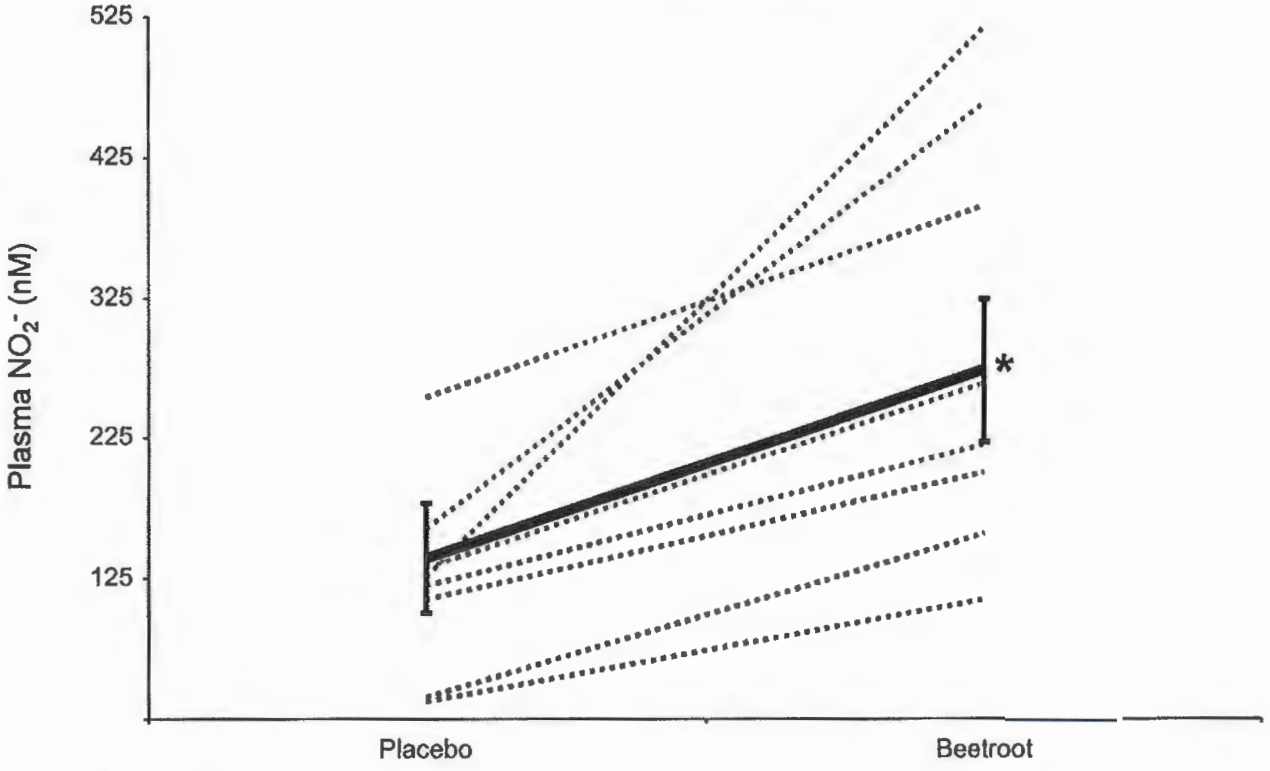
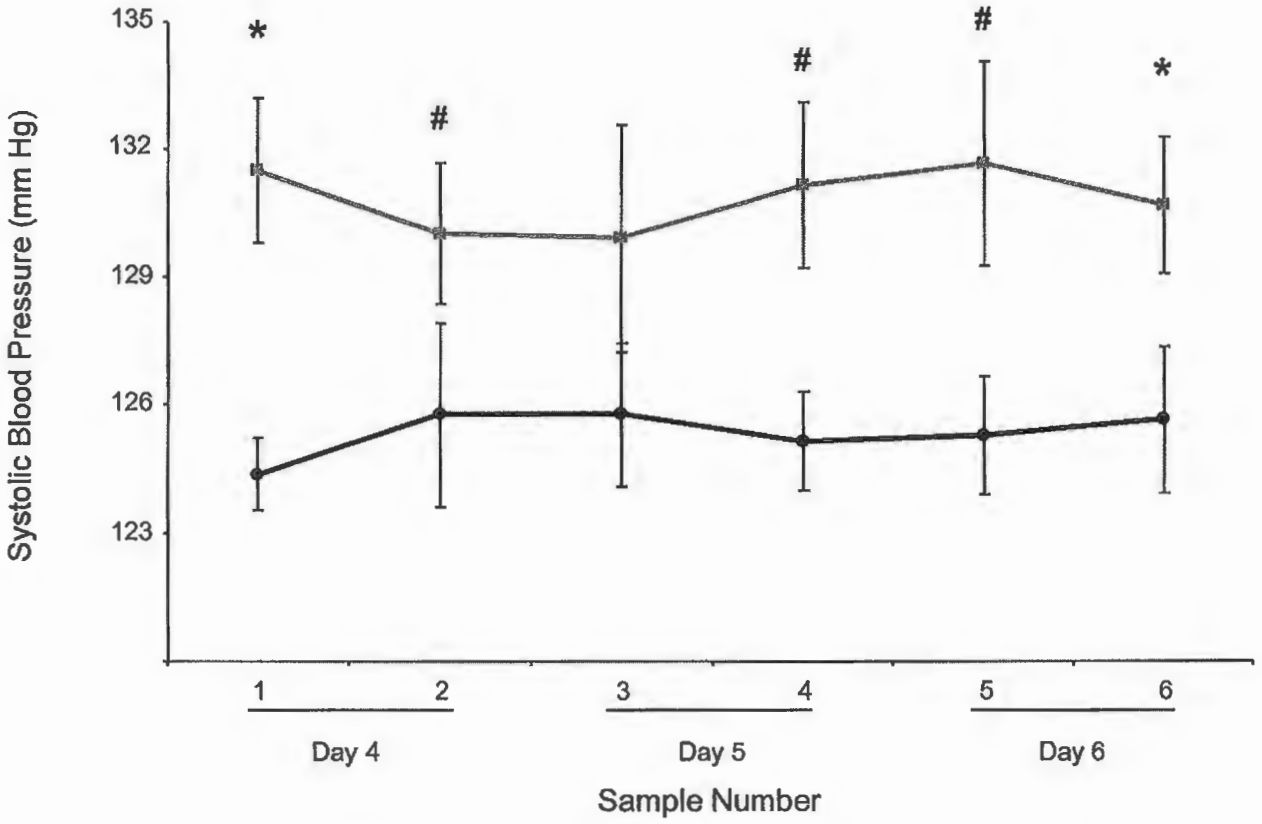

525
425
325
225
125
Plasma NO_2^- (nM)
*
Placebo
Beetroot

Figure 1


Systolic Blood Pressure (mm Hg)
135
132
129
126
123
*
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*
1
2
3
4
5
6
Day 4
Day 5
Day 6
Sample Number

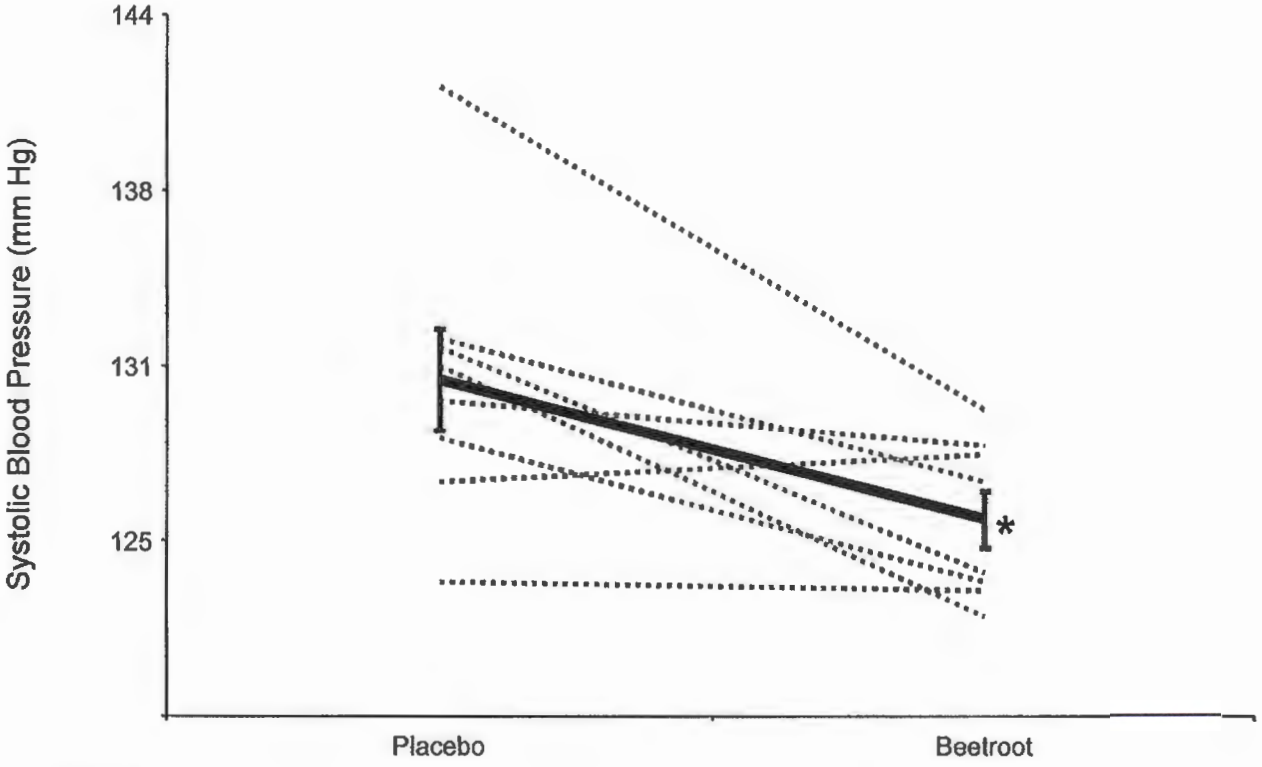
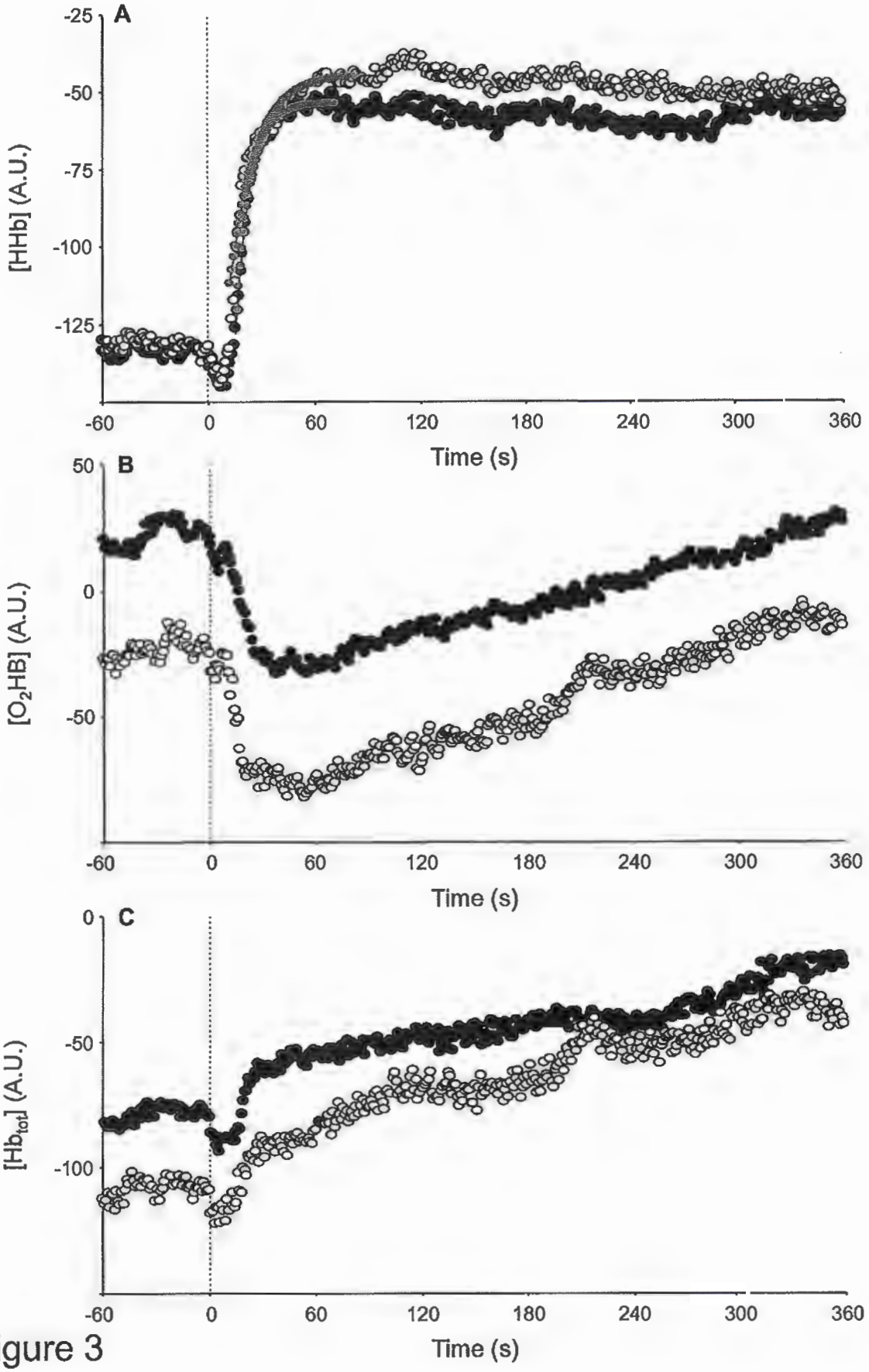

Systolic Blood Pressure (mm Hg)
144
138
131
125
*
Placebo
Beetroot

Figure 2


A
-25
-50
-75
-100
-125
[HHb] (A.U.)
-60
0
60
120
180
240
300
360
Time (s)
B
50
0
-50
[O2HB] (A.U.)
-60
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60
120
180
240
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Time (s)
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[Hbtot] (A.U.)
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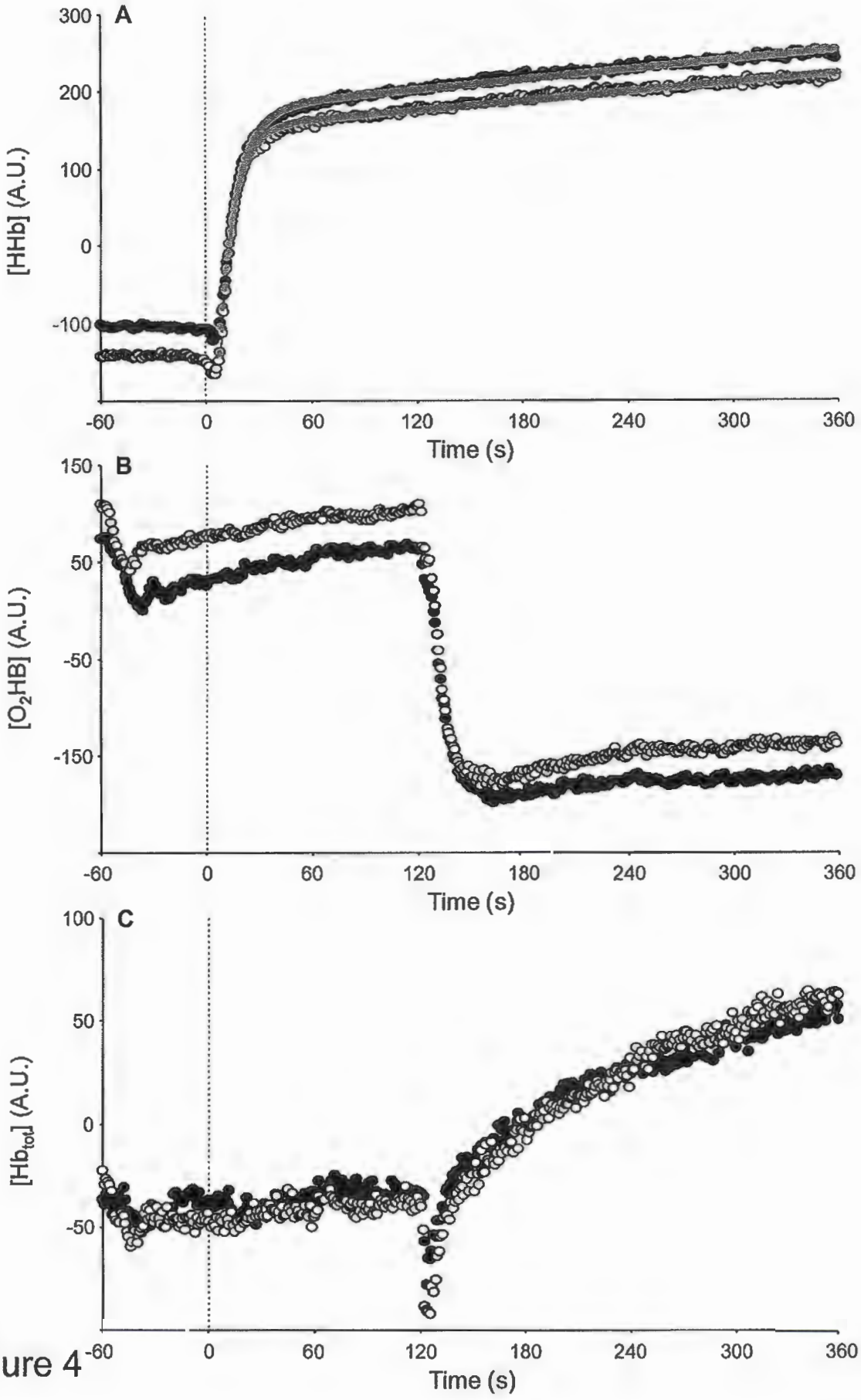
360
Time (s)

Figure 3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-100
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Time (s)
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150
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[O2HB] (A.U.)
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Time (s)
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100
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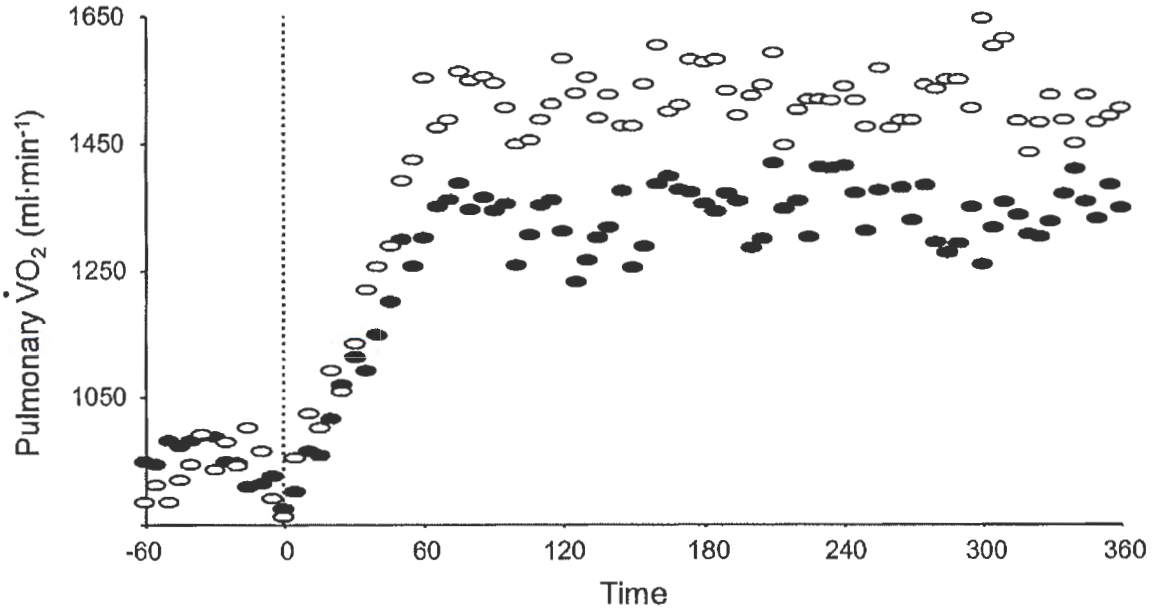
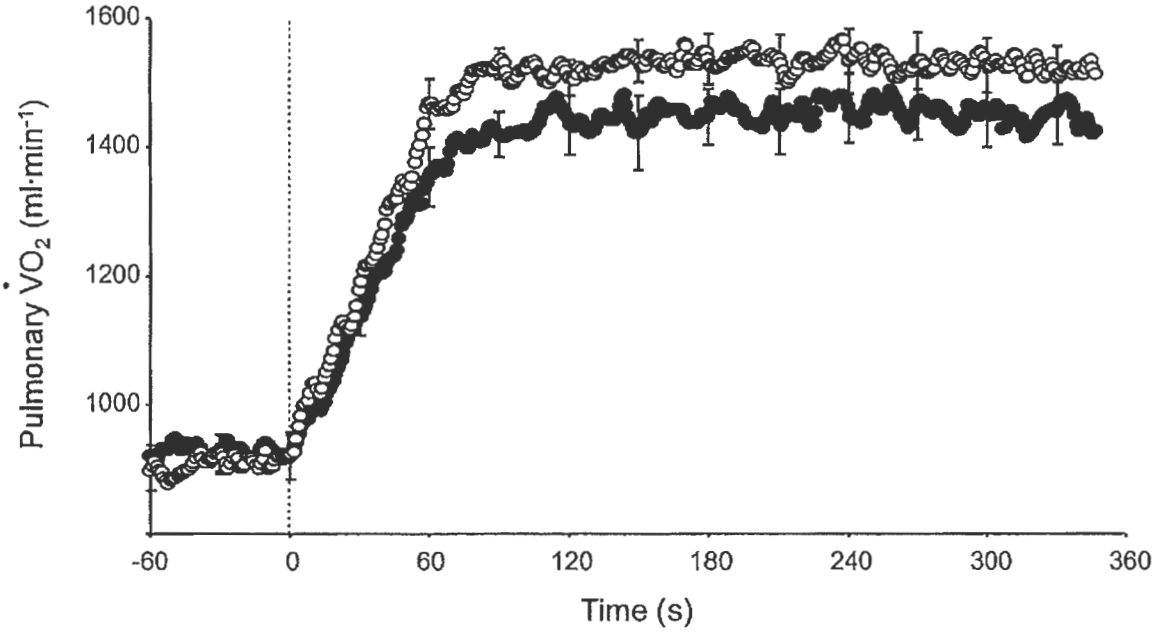
300
360
Time (s)

Figure 4


1650
1450
1250
1050
Pulmonary $\dot{V}O_2$ (ml·min^{-1})
-60
0
60
120
180
240
300
360
Time


1600
1400
1200
1000
Pulmonary $\dot{V}O_2$ (ml·min^{-1})
-60
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60
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180
240
300
360
Time (s)

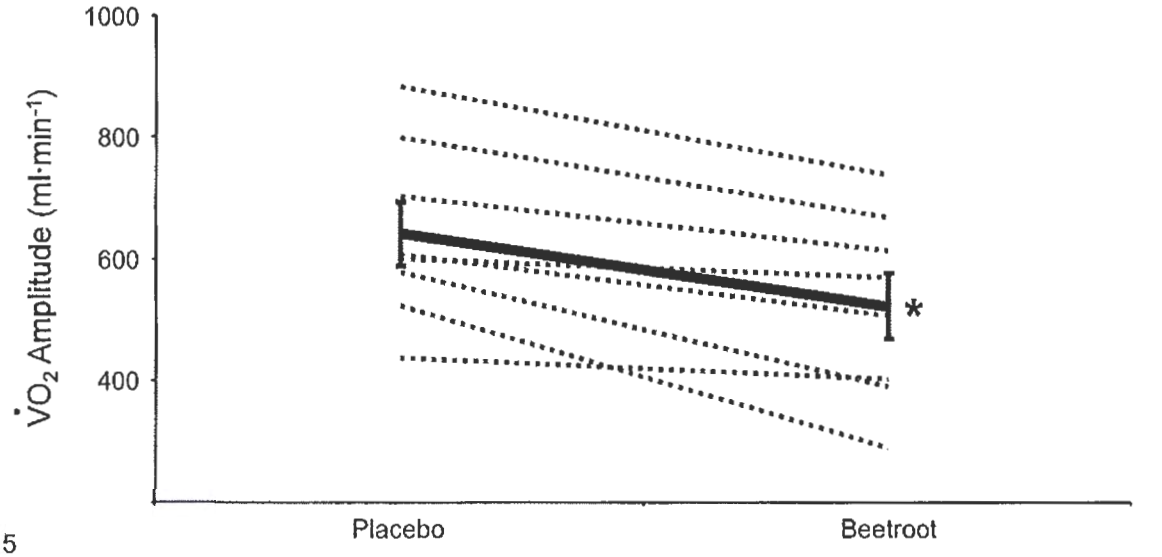
1000
800
600
400
$\dot{V}O_2$ Amplitude (ml·min^{-1})
*
Placebo
Beetroot

Figure 5

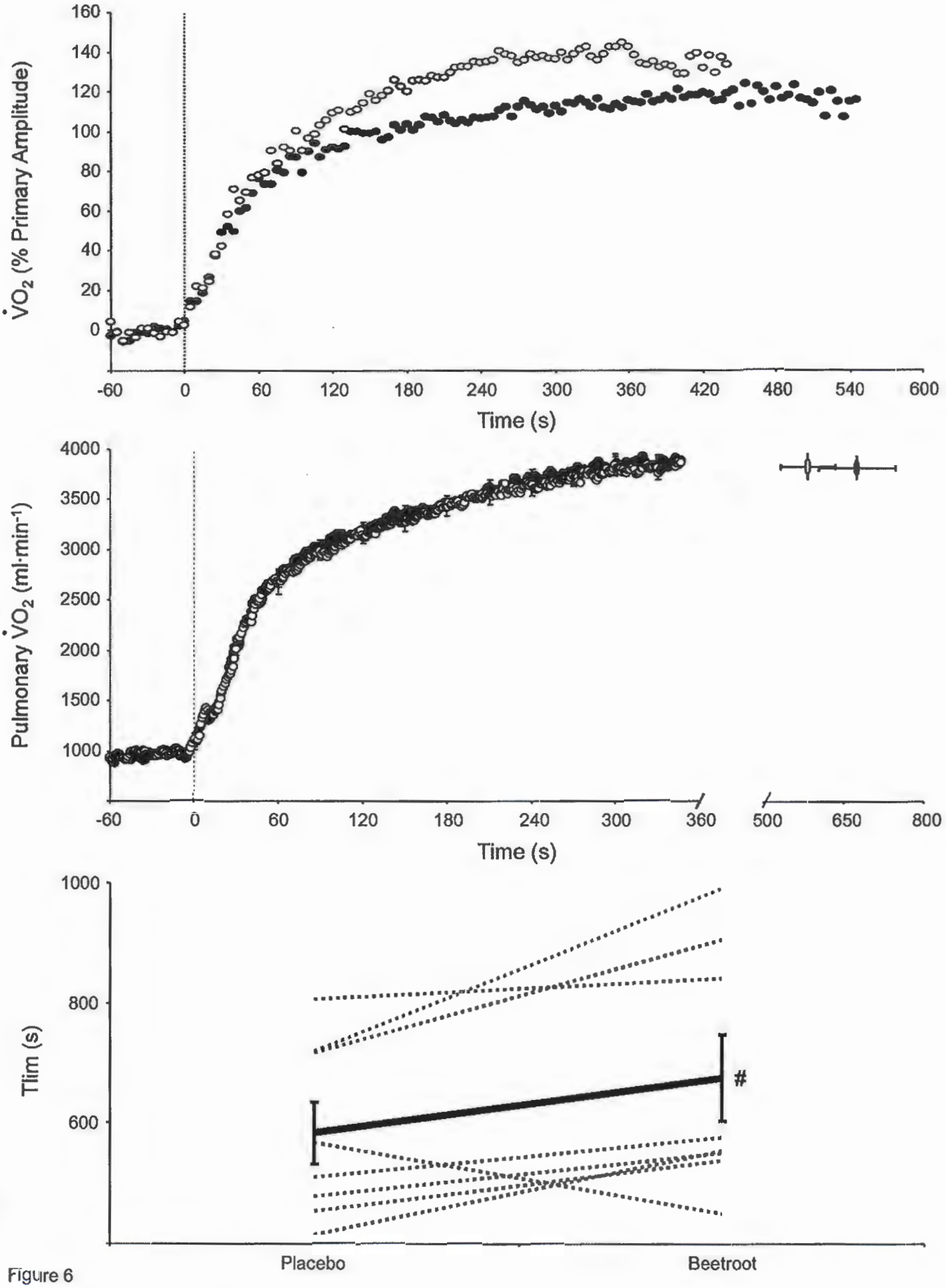
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Figure 6

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Amino Acids

Creatine supplementation reduces plasma levels of pro-inflammatory cytokines and PGE_2 after a half-ironman competition

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Summary. *Objective.* The effect of creatine supplementation upon plasma levels of pro-inflammatory cytokines: Interleukin (IL) 1β and IL-6, Tumor Necrosis Factor α (TNFα), and Interferon α (INFα) and Prostaglandin E_2 (PGE_2) after a half-ironman competition were investigated.

Methods. Eleven triathletes, each with at least three years experience of participation in this sport were randomly divided between the control and experimental groups. During 5 days prior to competition, the control group ($n=6$) was supplemented with carbohydrate ($20\,g \cdot d^{-1}$) whereas the experimental group ($n=5$) received creatine ($20\,g \cdot d^{-1}$) in a double-blind trial. Blood samples were collected 48 h before and 24 and 48 h after competition and were used for the measurement of cytokines and PGE_2.

Results. Forty-eight hours prior to competition there was no difference between groups in the plasma concentrations ($pg \cdot ml^{-1}$, mean ± SEM) of IL-6 (7.08 ± 0.63), TNFα (76.50 ± 5.60), INFα (18.32 ± 1.20), IL-1β (23.42 ± 5.52), and PGE_2 (39.71 ± 3.8). Twenty-four and 48 h after competition plasma levels of TNFα, INFα, IL-1β and PGE_2 were significantly increased ($P<0.05$) in both groups. However, the increases in these were markedly reduced following creatine supplementation. An increase in plasma IL-6 was observed only after 24 h and, in this case, there was no difference between the two groups.

Conclusion. Creatine supplementation before a long distance triathlon competition may reduce the inflammatory response induced by this form of strenuous of exercise.

Keywords: Long distance triathlon – Muscle inflammation – Muscle damage – Eccentric contraction – Pro-inflammatory cytokines

Introduction

The growing interest in triathlon is based on the original structure of the sport itself and in the wide variety of distances offered to participants, ranging from sprint to ironman distance, and lasting from 1 to 10 h, respectively. The triathlon comprises sequentially of swimming, cycling, and running. The leading causes of trauma during and after endurance exercise are a) eccentric muscle contractions, as in the long distance triathlon, b) the impact of the extremities against the ground and c) the number of repetitions of the same movement (Bansil et al., 1985). Delayed onset muscle soreness (DOMS) develops 24–48 h after as a consequence of prolonged eccentric muscle contraction and the strenuous nature of events such as long distance triathlon. (Bansil et al., 1985; Milles and Clarkson, 1994; Egermann, et al., 2003; Volek and Rawson, 2004). The discomfort experienced by athletes is generally accompanied by prolonged muscle-strength loss, a reduced range of motion, and high levels of creatine kinase activity in the blood (Milles and Clarkson, 1994). Muscle swelling and the sensation of DOMS suggests that physical exercise causes muscle inflammation, especially if the exercise is strenuous and/or involves eccentric contractions as in marathon or long distance triathlon (Malloch and Taunton, 2000; Malm, 2001; Clarkson and Hubal, 2002). The passive elements (connective tissue and muscle fibers) can absorb strain and this ability increases by as much as 100% after muscle activation (Malloch and Taunton, 2000). However, when muscle activation is compromised, as during fatigue caused by endurance exercise, the ability to absorb strain is reduced, increasing muscle vulnerability to trauma (Malloch and Taunton, 2000).

The plasma levels of IL-1β, IL-6, TNFα, INFα, and C reactive protein are known to increase during strenuous exercise (Malm, 2001; Clarkson and Hubal, 2002; Nosaka and Newton, 2002). Exercise also induces an increase in prostaglandin E_2 (PGE_2) production, as part of the inflammatory response triggered by micro trauma occurring

[†] In memoriam.

in the skeletal muscles (Bansil et al., 1985; Smith and Miles, 2000). There is an increase of PGE_2 synthesis by infiltrating macrophages in the inflamed muscle, and this is a key mediator of pain 24–48 h after an exercise session (Volek and Rawson, 2004).

Creatine improves performance during repeated bouts of high intense exercise when supplemented for a short period of time (Mujika and Padilla, 1997; Lawler et al., 2002). The typical programme of creatine intake consists of 20 g per day for 5–7 days followed by a maintenance load of 3–5 g per day (Harris et al., 1992; Milles and Clarkson, 1994; Bemben and Lamont, 2005). The greatest uptake of creatine by the muscle occurs during the initial stages of the loading regime (Harris et al., 1992). Exercise seems to enhance the uptake of creatine (Harris et al., 1992), especially if ingested with a carbohydrate drink after exertion (Terjung et al., 2000). Oral intake of creatine is known to increase glycogen concentrations in human skeletal muscle by up to 40% (Op T'Eijnde et al., 2001). Creatine supplementation also causes weight gain by increasing the retention of intracellular water, increasing muscle cell volume (Demant and Rhodes, 1999; Terjung et al., 2000). Moreover, creatine supplementation has been reported to maintain muscle integrity, reducing muscle damage and inflammatory responses and attenuating the increase in plasma PGE_2 levels (Santos et al., 2004).

In the present report we investigated the effects of creatine supplementation upon plasma levels of pro-inflammatory cytokines (IL-1β, IL-6, TNFα and INFα) and PGE_2 after a half-ironman competition consisting of 1.9 km of swimming, 90 km of cycling and 21 km of running. In summary, creatine supplementation for five days prior to the competition was shown to reduce the plasma levels of the most cytokines evaluated, except for IL-6, and PGE_2 even after 48 h of the competition.

Materials and methods

Subjects and experimental

The experimental protocol was approved by the Ethics Committee of the Institute of Biomedical Sciences, University of Sao Paulo, São Paulo, Brazil. The subjects were selected from a group of non-smoking athletes who were in training for a half-ironman triathlon (Long Distance Triathlon Brazilian Championship). None of the athletes had a history of consuming forbidden drugs or anti-inflammatory medications and did not take creatine as a supplement. The athletes average age was 40.3 years, range 34–56 years. The range in self-reported personal best performance in a half-ironman competition was 266–311 min. In the present study the athletes completed the half-ironman triathlon competition in the range of 280–329 min (4 h 40 min–5 h 29 min – Table 1). All athletes had previously participated in at least three half-ironman triathlon competitions.

After signing an informed consent form, eleven male athletes were randomly divided into the control (Pl – $n = 6$) and experimental (Cr – $n = 5$) groups. During five days prior to the competition, athletes from the experimental group received 20 g per day of creatine monohydrate divided in two equal doses. These were ingested at 10 am and 4 pm. Athletes from the control group similarly received 20 g per day of carbohydrate in place of creatine. The creatine or carbohydrate supplementation was supplied to athletes as a ready-to-consume powder mixture containing maltodextrin 50 g. The athletes from both groups were instructed to dilute the powder with the same volume of water just before the ingestion. The mixture offered for both groups had similar volume, flavour and color to avoid identification of the supplements. The athletes were allowed to drink and eat normally during the five days that preceded the competition. All experiments were conducted as a double-blind trial. The race started at 8:30 am with 30 °C and 90% relative-humidity. Conditions at the end of the competition were 38 °C and 80% RH. All athletes finished the race within 5% of their best personal time for that distance.

Table 1. Physical data and degree of training (triathletes, $n = 11$)

	Mean ± SEM	Range
Age (yrs)	40.3 ± 2.18	34–56
Weight (kg)	76.3 ± 2.29	63.6–85.0
Height (cm)	178.2 ± 2.04	170–190
Personal best (min)	290.5 ± 5.05	266–311
Regular triathlon (yrs)	5.3 ± 0.80	3–10
Half-ironman time (min)	302.2 ± 4.52	280–329
Training (time/wk)	177.0 ± 12.8	120–240
VO_2 max. ($ml/kg \cdot min^{-1}$)	52.3 ± 1.34	45.3–58.0

Physical data and the degree of training showed that the triathletes were homogeneous in terms of running experience and performance in a half-ironman competition (Table 1).

Blood sampling

Blood samples (20 ml) were collected from an antecubital vein 48 h before the start, and 24 and 48 h after the end of the competition, into sterile heparinized glass tubes. Blood samples were centrifuged at $650 \times g$ for 15 min and plasma was kept at −70 °C for one week until analysed for cytokines (IL-1, IL-6, TNFα and INFα) and prostaglandin E_2 (PGE_2).

Measurements of the cytokines and PGE_2

Plasma IL-1β, IL-6, TNFα, INFα and PGE_2 concentrations were determined using commercially available ELISA-kits (Biotrak – cellular communication assays, Amersham Pharmacia biotech, Little Chalfont Buckinghamshire, UK).

Statistical analysis

The results were compared using two-way ANOVA. Group means were further compared using the post-hoc test of Bonferroni. A level of significance of at least $p < 0.05$ was chosen for all comparisons. All data were analyzed using Graph Pad Prism program and graph package (V4.0, Graph Pad Inc., San Diego, CA, USA). The results are presented as mean ± SEM.

Results

Forty-eight hours before the half-ironman competition plasma concentrations (mean ± SEM) of IL-6 ($7.08 \pm 0.63\,pg \cdot ml^{-1}$), TNFα ($76.50 \pm 5.60\,pg \cdot ml^{-1}$), INFα ($18.32 \pm 1.20\,pg \cdot ml^{-1}$), IL-1β ($23.42 \pm 3.52\,pg \cdot ml^{-1}$), and PGE_2 ($39.71 \pm 3.8\,pg \cdot ml^{-1}$) were within the normal

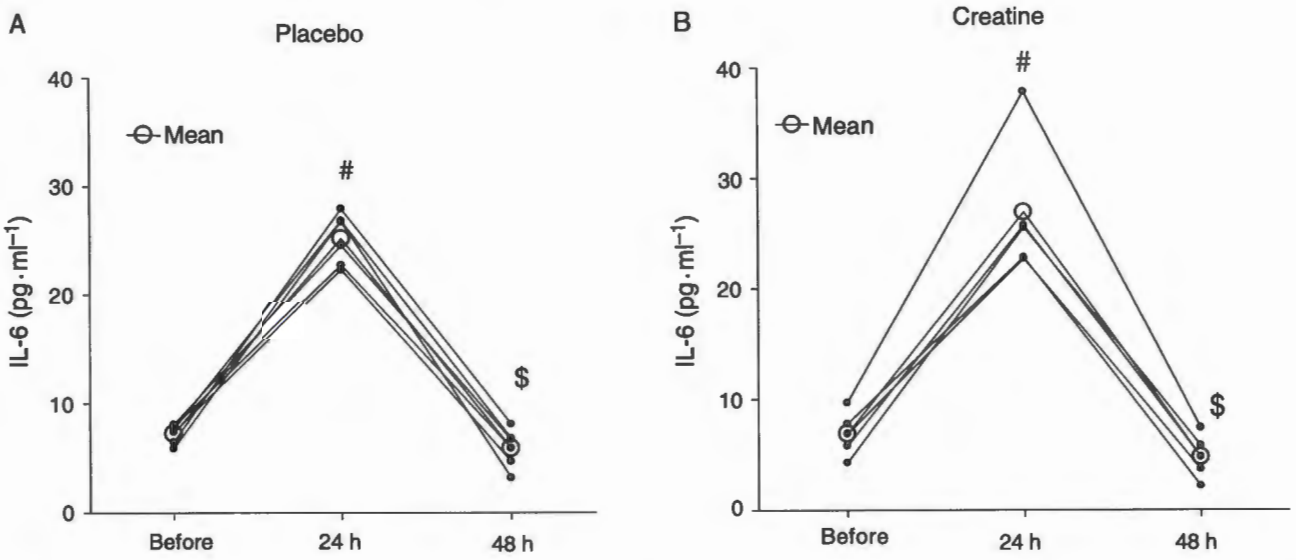

Fig. 1. Interleukin-6 (IL-6) plasma concentration. IL-6 levels was determined 48 h before, and 24 and 48 h after the end of the competition in plasma collected from triathletes that received either placebo (Pl, $n = 6$) or creatine supplementation (Cr, $n = 5$). The results are expressed as mean. Standard errors of the means were always lower of the 20% of the mean value. $^{*}p < 0.05$ for comparison with the creatine supplemented group. $^{\#}p < 0.05$ for comparison with the data before the competition in both groups. $^{\$}p < 0.05$ for comparison between 48 and 24 h in both groups

expected range (Santos et al., 2004). There were no significant differences between the control and experimental groups.

Following competition plasma IL-6 at 24 h was increased 3.5-fold (7.25 ± 0.35 vs. 25.15 ± 0.88; $p < 0.05$) and 3.9-fold (6.90 ± 0.91 vs. 29.90 ± 2.80; $p < 0.05$) in the control and experimental groups, respectively (Fig. 1). However, by 48 h IL-6 had fallen back to 76.5% (5.90 ± 0.71; $p < 0.05$) and 82.2% (4.78 ± 0.89; $p < 0.05$) of the pre-competition concentration in the control and experimental groups, respectively. There was no effect of creatine supplementation on the response of plasma IL-6 to exercise under the conditions of this study.

Plasma TNFα was increased in the control group 3.5-fold at 24 h to 287.48 ± 8.50 ($p < 0.05$) and 4-fold at 48 h to 324.40 ± 9.94 ($p < 0.05$) compared to the concentration

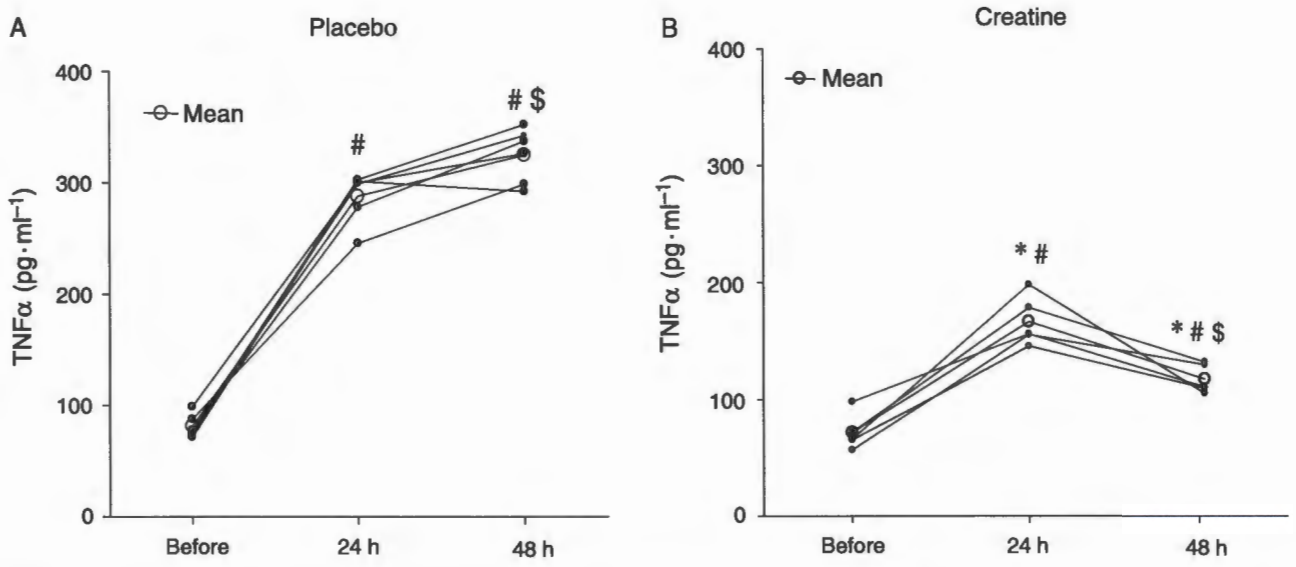

Fig. 2. Tumor Necrosis Factor α (TNFα) plasma concentration. TNFα levels was determined 48 h before, and 24 and 48 h after the end of the competition in plasma collected from triathletes that received either placebo (Pl, $n = 6$) or creatine supplementation (Cr, $n = 5$). The results are expressed as mean. Standard errors of the means were always lower of the 10% of the mean value. $^{*}p < 0.05$ for comparison with the creatine supplemented group. $^{\#}p < 0.05$ for comparison with the data before the competition in both groups. $^{\$}p < 0.05$ for comparison between 48 and 24 h in both groups

before competition (81.37 ± 4.15) (Fig. 2). The further increase from 24 to 48 h was significant. Creatine supplementation significantly reduced the increases in TNFα at 24 and 48 h by 42 and 64%. Concentrations at these times (166.64 ± 9.54 and 117.22 ± 5.55) were significantly lower ($p < 0.05$) than those in the control group (Fig. 2). The decrease from 24 to 48 h was significant.

Plasma INFα was increased in the control group 16.5-fold at 24 h to 296.93 ± 8.29 ($p < 0.05$) and 16.3-fold at 48 h to 293.33 ± 5.39 ($p < 0.05$) compared to the concentration before competition (18.05 ± 1.30) (Fig. 3). The change from 24 to 48 h was not significant. Creatine supplementation significantly reduced ($p < 0.05$) the increase in INFα at 24 and 48 h by 50.5 and 80.1%. Concentrations at these times (147.08 ± 2.46 and 58.24 ± 6.10) were significantly lower than the corresponding concentrations in the control group (Fig. 3). The decrease from 24 to 48 h was significant ($P < 0.05$) by 60.4%.

Plasma IL1-β was increased in the control group 6.9-fold at 24 h to 157.50 ± 7.74 ($p < 0.05$) and 6.9-fold at

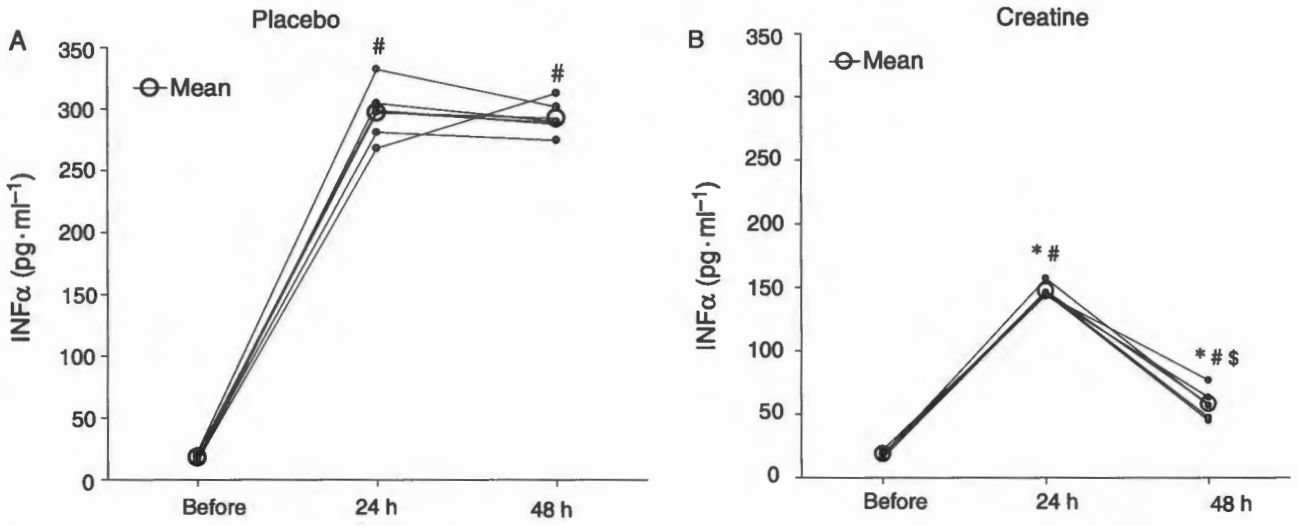

Fig. 3. Interferonα (INFα) plasma concentration. INFα levels was determined 48 h before, and 24 and 48 h after the end of the competition in plasma collected from triathletes that received either placebo (**A**, Pl, $n = 6$) or creatine supplementation (**B**, Cr, $n = 5$). The results are expressed as mean. Standard errors of the means were always lower of the 15% of the mean value. * $p < 0.05$ for comparison with the creatine supplemented group. # $p < 0.05$ for comparison with the data before the competition in both groups. $ $p < 0.05$ for comparison between 48 and 24 h in both groups

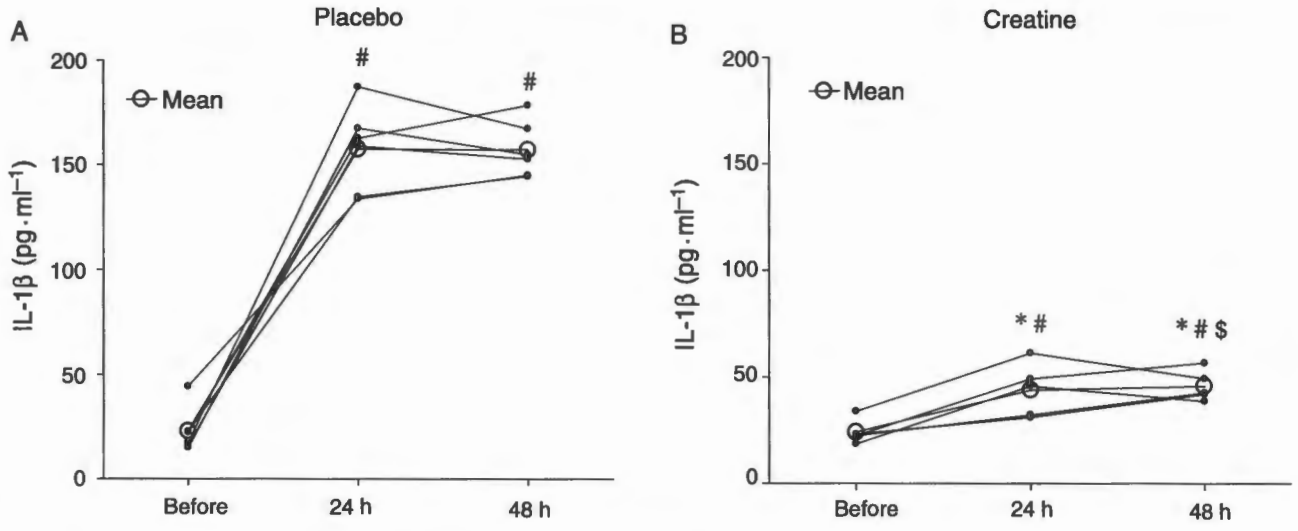

Fig. 4. Interleukin-1 β (IL-1β) plasma concentration. IL-1β levels was determined 48 h before, and 24 and 48 h after the end of the competition in plasma collected from triathletes that received either placebo (**A**, Pl, $n = 6$) or creatine supplementation (**B**, Cr, $n = 5$). The results are expressed as mean. Standard errors of the means were always lower of the 20% of the mean value. * $p < 0.05$ for comparison with the creatine supplemented group. # $p < 0.05$ for comparison with the data before the competition in both groups. $ $p < 0.05$ for comparison between 48 and 24 h in both groups

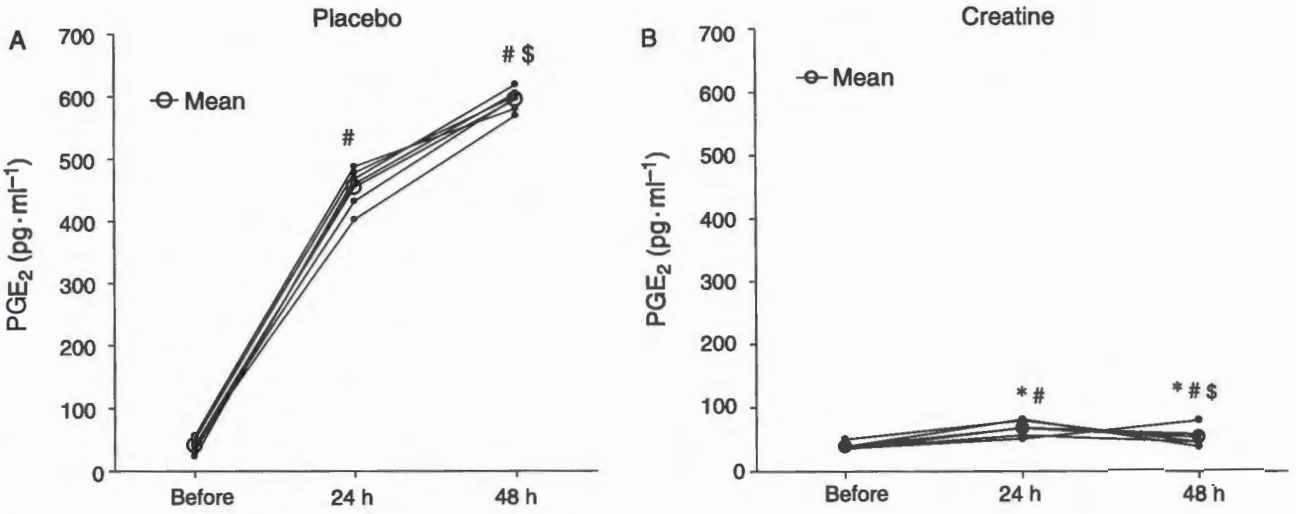

Fig. 5. Prostaglandin E_2 (PGE_2) plasma concentration. PGE_2 levels was determined 48 h before, and 24 and 48 h after the end of the competition in plasma collected from triathletes that received either placebo (**A**, Pl, $n = 6$) or creatine supplementation (**B**, Cr, $n = 5$). The results are expressed as mean. Standard errors of the means were always lower of the 15% of the mean value. $^{*}p < 0.05$ for comparison with the creatine supplemented group. $^{\#}p < 0.05$ for comparison with the data before the competition in both groups. $^{\$}p < 0.05$ for comparison between 48 and 24 h in both groups

48 h to 157.15 ± 5.44 ($p < 0.05$) compared to the concentration before competition (22.82 ± 4.47) (Fig. 4). The change from 24 to 48 h was not significant. Creatine supplementation significantly ($p < 0.05$) reduced the increases in IL1-β at 24 and 48 h by 72 and 71%. Concentrations at these times (43.86 ± 5.67 and 45.82 ± 3.23) were significantly lower than the corresponding concentrations in the control group ($p < 0.05$) (Fig. 4).

Plasma PGE_2 was increased in the control group 11-fold at 24 h to 454.37 ± 11.96 ($p < 0.05$) and 14.4-fold at 48 h to 594.7 ± 7.3 ($p < 0.05$) compared to the concentration before competition (41.22 ± 4.79) (Fig. 5). The change from 24 to 48 h was significant. Creatine supplementation significantly reduced ($p < 0.05$) the increase in PGE_2 at 24 and 48 h by 85.5 and 91%. Concentrations at these times (65.70 ± 5.85 and 52.56 ± 7.10) were significantly lower than the corresponding concentrations in the control group ($p < 0.05$) (Fig. 5).

Discussion

The major finding of the present report is that athletes receiving creatine supplementation during the five day period that precedes the half-ironman shows significantly lower levels of pro-inflammatory mediators, such as TNFα, INFα, IL1-β and PGE_2, at 24 and 48 h after the competition.

In response to an infectious agent or a nonspecific form of tissue injury, the host shows an acute inflammatory reaction in the affected tissue. Polymorphonuclear neutrophils (PMNs), mast cells, and macrophages are important cellular components of this inflammatory process. Pro-inflammatory mediators such as prostaglandins are released from PMNs and/or macrophages and various cytokines are released from macrophages and/or lymphocytes. The pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) evaluated herein are derived mainly from macrophages and have many systemic and metabolic effects. Of the major importance is the impact of these cytokines on the liver, leading to production of a number of proteins generally named *acute phase proteins* (APP). These events are collectively called as the acute phase response and are characterized by fever, leukocytosis, decreased appetite, altered sleep patterns, and malaise, also referred to as sickness behavior. The early or alarm cytokines are IL-1β and TNFα, which act locally on fibroblasts and endothelial cells to induce the production of other cytokines such as IL-6 acting centrally through the induction of prostaglandins to cause fever and the sickness behavior (Baumann and Glaudie, 1994). Both primary and secondary cytokines initiate other cytokine cascades that make up the acute phase response. For instance, the APP produced by the liver have a wide range of activities, such as neutralizing inflammatory agents to minimize the extent of the damage or participating in the repair of tissues that contribute to the host defense (Steel and Witehead, 1994).

The production of pro-inflammatory cytokines, such as IL1β, IL-6, TNFα, and INFα, is increased during intense and prolonged exercise (Mackinnon, 1999). These type of exercise may cause damage to and inflammation within skeletal muscle (Malm, 2001). The increase in IL-1β, and

TNFα levels may be a result of high plasma levels of stress hormones such as catecholamines and corticosteroids (Cupps and Fauci, 1982; Nieman and Nehlsen-Cannarella, 1991); the plasma levels of these hormones rise dramatically during physical exercise (Farrel et al., 1983; Mackinnon, 1999). The overall damage in tissues is characterized by movement of fluid and plasma proteins. Neutrophils represent the first wave of infiltrating cells, followed by monocytes (Smith and Miles, 2000). In addition, the overt signs and symptoms of the inflamed tissue include swelling, redness, hest, pain, and loss of or reduced function (Smith and Miles, 2000). Skeletal muscle injury manifests within few hours, increasing as exercise progresses, and it is still apparent after completion of the exercise session (Farber et al., 1991). Thus, considering that *delay onset muscle soreness* (DOMS) develops 24–48 h after strenuous exercise as a consequence of eccentric muscle contraction or strenuous endurance events, blood samples collected within this period should reflect the plasma levels of pro-inflammatory mediators (Thompson et al., 1997; Egermann et al., 2003).

As observed herein, in the half-ironman triathlon competition, the plasma levels of pro-inflammatory cytokines markedly increased after the competition. Another aspect related to the muscle inflammation process is the increase in plasma levels of PGE_2 observed 48 h after 30 km race in marathon runners (Santos et al., 2004). PGE_2 is actively synthesized by macrophages upon exposure to an inflammatory environment and it has been implicated in the pain following exertion (Thompson et al., 1997). This fact is in agreement with the increase in PGE_2 plasma concentrations observed in the present study.

Data from muscle biopsies indicate that IL-1β and IL-6 are produced within skeletal muscle during and after exercise being associated with muscle damage (Canonn et al., 1989; Rohde et al., 1997). Inflammatory activity within skeletal muscle can also be driven by the local endothelial cells. IL-1β has been implicated in muscle proteolysis and repair following injury/inflammation (Mackinnon, 1999). However, muscle contraction, even in the absence of markers of muscle damage, rapidly increases IL-6 mRNA expression as observed in skeletal muscle biopsy samples (Smith and Miles, 2000; Pedersen et al., 2003; Bemben and Lamont, 2005). IL-6 production by skeletal muscle varies with exercise intensity, duration, the mass of muscle recruited, and endurance capacity (Pedersen et al., 2003, 2004; Febbraio et al., 2004). The release of this cytokine by skeletal muscle may play a role to mobilize substrates for energy production (O'Toole et al., 1989; Op T'Eijnde et al., 2001). Recent study has shown that IL-6 is released from skeletal muscle during exercise and that carbohydrate ingestion attenuates the increase in the production of this cytokine during both running and cycling (Pedersen and Febbraio, 2005).

Skeletal muscle contraction is a powerful stimulus for glucose disposal and uptake leading to hypoglycemia if the endogenous glucose production and output from the liver are not stimulated at the same extent during exercise (Pedersen and Febbraio, 2005). IL-6 influences glucose homeostasis during exercise and provides potential new insights into factors that mediate glucose production and disposal. There is strong evidence that IL-6 may also affect lipid metabolism in humans resulting in lypolysis and fatty acid oxidation (Pedersen and Febbraio, 2005).

The increase in IL-6 after 24 h may be important to maintain blood glucose levels, to inhibit TNFα production and to increase insulin sensitivity protecting against certain disorders, such as type 2 diabetes (Pedersen and Hoffman-Goetz, 2000). Usually IL-6 is referred as an "inflammation-responsive" cytokine rather than a pro-inflammatory cytokine as IL-6 directly do not induce inflammation. Additionally, proinflammatory cytokines such as IL-1β and TNFα have been shown to induce PGE_2 synthesis in endothelial cells, smooth muscle cells, and skeletal muscle. IL-6 production and release plasma seems to precede neutrophil and macrophage accumulation in the muscle, as a high level of IL-6 is found immediately after an exhaustive exercise bout (Pedersen and Hoffman-Goetz, 2000).

The plasma levels of TNF-α is also elevated in the inflammation process, however, chronic muscular activity down regulates this cytokine expression in contracting skeletal muscle of elderly humans (Pedersen et al., 2004; Pedersen and Febbraio, 2005).

The creatine supplementation tested in the present study was able to reduce the increase in plasma levels of the pro-inflammatory cytokines (IL1β, TNFα, and INFα) in addition to PGE_2, when compared with the placebo group, in both situations (24 and 48 h after competition). In spite of this, however, the mechanism involved remains to be determined. There is possible that creatine supplementation may reduce muscle cell death and as consequence the inflammatory process as whole. The absence of effect of creatine supplementation on IL-6 plasma may reflect on adjustment of the changes induced in the remaining cytokines.

In conclusion, the results presented herein suggest that creatine supplementation during a short period of five days before the half-ironman triathlon may attenuate the increase in plasma levels of pro-inflammatory cytokines and PGE_2. Further experiments are now required to investigate if this anti-inflammatory response is due to systemic

and/or local effect of creatine on leukocytes (macrophages) and/or the exercised muscle.

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Creatine Supplementation Normalizes Mutagenesis of Mitochondrial DNA as Well as Functional Consequences

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Mutations of mitochondrial (mt) DNA play a role in neurodegeneration, normal aging, premature aging of the skin (photoaging), and tumors. We and others could demonstrate that mtDNA mutations can be induced in skin cells *in vitro* and in normal human skin *in vivo* by repetitive, sublethal ultraviolet (UV)-A-irradiation. These mutations are mediated by singlet oxygen and persist in human skin as long-term biomarkers of UV exposure. Although mtDNA exclusively encodes for the respiratory chain, involvement of the energy metabolism in mtDNA mutagenesis and a protective role of the energy precursor creatine have thus far not been shown. We assessed the amount of a marker mutation of mtDNA, the so-called common deletion, by real-time PCR. Induction of the common deletion was paralleled by a measurable decrease of oxygen consumption, mitochondrial membrane potential, and ATP content, as well as an increase of matrix metalloproteinase-1. Mitochondrial mutagenesis as well as functional consequences could be normalized by increasing intracellular creatine levels. These data indicate that increase of the energy precursor creatine protects from functionally relevant, aging-associated mutations of mitochondrial DNA.

Key words: aging/functional relevance/matrix metalloproteinase/oxidative damage/photoaging
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Mutations of mitochondrial (mt) DNA have initially been reported to play a causative role in inherited neurological diseases (reviewed in DiMauro and Schon, 2003)). Further publications extended these findings to acquired neurodegenerative diseases (Holt *et al*, 1988; Cao *et al*, 2001; Wanrooij *et al*, 2004), the normal aging process (Wallace, 2001; Cortopassi, 2002; Hofhaus *et al*, 2003), and premature aging of the skin, also called photoaging (Birch-Machin *et al*, 1998; Berneburg *et al*, 2000). In addition to this, mtDNA mutations have also been reported to be increased in several types of tumors affecting the colon, bladder, lung, breast, kidney, head, and neck (Heerdt *et al*, 1994; Burgart *et al*, 1995; Habano *et al*, 1998, 1999, 2000; Polyak *et al*, 1998; Fliss *et al*, 2000; Parrella *et al*, 2001); however, the relevance of these mutations remains to be established. We and others have previously shown that repetitive, sublethal exposure to ultraviolet A light (UVA) irradiation at doses acquired during a regular summer holiday induces mutations of mtDNA in keratinocytes and fibroblasts in a singlet oxygen-dependent fashion as well as in normal human skin (Yang *et al*, 1994, 1995; Birch-Machin *et al*, 1998; Berneburg *et al*, 1999, 2000, 2004; Koch *et al*, 2001). Furthermore, we could show that, once induced, these mutations persist for at least 16 months in UV-exposed skin. Therefore, these mutations represent long-term biomarkers for UV-exposure in human skin (Berneburg *et al*, 2004). There is a large body of evidence indicating that mitochondrial function decreases when mutations of mtDNA increase (Wallace, 2001; Cortopassi, 2002; Stuart *et al*, 2004) and a vicious cycle has been hypothesized in which oxidative stress induces mutations of mtDNA leading to a defective respiratory chain, in turn leading to reduced energy production (Lenaz, 1998; DiMauro *et al*, 2002; Jacobs, 2003; Pak *et al*, 2003). Cells contain mutant and wild-type mtDNA molecules at the same time, also called heteroplasmy (Hofhaus *et al*, 2003). Therefore, in order to become biochemically relevant, the ratio of mutant molecules to wild-type molecules has to exceed a threshold that differs from tissue to tissue. This is one of the reasons why thus far it could not be shown that repetitively UV-induced mtDNA mutations may reach levels of functional relevance and whether supplementation with an energy precursor such as creatine can protect from mtDNA mutagenesis and resulting functional consequences in normal human fibroblasts. In this study, sublethal repetitive UVA-exposure was indeed able to reduce cellular oxygen consumption, mitochondrial membrane potential $\delta\psi$, and ATP content as parameters for mitochondrial function. Furthermore, UV-exposure also led to induction of matrix-metalloproteinase (MMP)-1, known to be involved in processes such as photoaging and carcinogenesis. Coincubation of cells in the presence of the energy precursor creatine

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; MMP-1, matrix-metalloproteinase-1; mt, mitochondria; ROS, reactive oxygen species; TIMP-1, tissue-specific inhibitor of MMP-1; UVA, ultraviolet A light

abrogated these effects in a manner independent from antioxidant effects of reactive oxygen species (ROS)-quenchers such as α-tocopherol. Our data suggest that mtDNA mutations induced by sublethal repetitive UV-exposure of a magnitude acquirable during a regular summer holiday suffices to result in functionally relevant changes and that creatine supplementation of cells is able to normalize mtDNA mutagenesis and functional impairment.

Results

We investigated whether cells containing the UVA-induced common deletion showed changes in functional parameters.

Generation of the common deletion in normal human fibroblasts by repetitive UVA-irradiation Normal human fibroblasts were repetitively exposed to UVA doses of 8 J per cm^2 three times daily as described previously (Berneburg *et al*, 1997, 1999, 2004; Koch *et al*, 2001; Eicker *et al*, 2003). Real-time PCR of total DNA extracts from normal human fibroblasts confirmed the induction of the common deletion over the course of three weeks as described previously (Fig 1*a*). Real-time PCR showed background levels of the common deletion in unirradiated samples as well as in cells that were exposed to 12 UVA-irradiations (1 wk). Two weeks of UVA-exposure (24 irradiations) led to the first detectable induction of the common deletion, which could be further increased after three weeks of UVA-exposure (36 irradiations). The amount of the common deletion relative to unirradiated controls were week 1, 95%, week 2, 2.2-fold; and week 3, 38-fold. Therefore, we could confirm the previously observed induction of the common deletion *in vitro* in normal human fibroblasts.

Reduction of oxygen consumption in fibroblasts carrying the common deletion Cellular oxygen consumption is an indication of mitochondrial function. We assessed the oxygen consumption of normal human fibroblasts containing the common deletion by a standard Clark-type electrode. Whereas sham-irradiated cells showed normal oxygen consumption for three weeks, values for UVA-exposed cells continuously decreased during the course of the irradiation regimen while the common deletion increased in these cells (Fig 1*b*). Control measurements in rho 0 cells containing no mtDNA showed no alteration of oxygen consumption with values not leaving baseline levels (data not shown).

Reduction of mitochondrial membrane potential (Δψ) Measurement of Δψ was carried out using the fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) that accumulates in the mitochondria with normal membrane potential where it fluoresces red. It remains outside the mitochondria with abnormal Δψ, and then showing green fluorescence (Smiley *et al*, 1991). Sham-irradiated fibroblasts showed abundant red fluorescence visualizing normal cytoplasmatically located mitochondria (Fig 1*c*). Repetitively UVA-exposed fibroblasts, carrying the common deletion, showed continuously decreasing red fluorescence with stable total mitochondrial protein content, indicating reduction of Δψ parallel to the

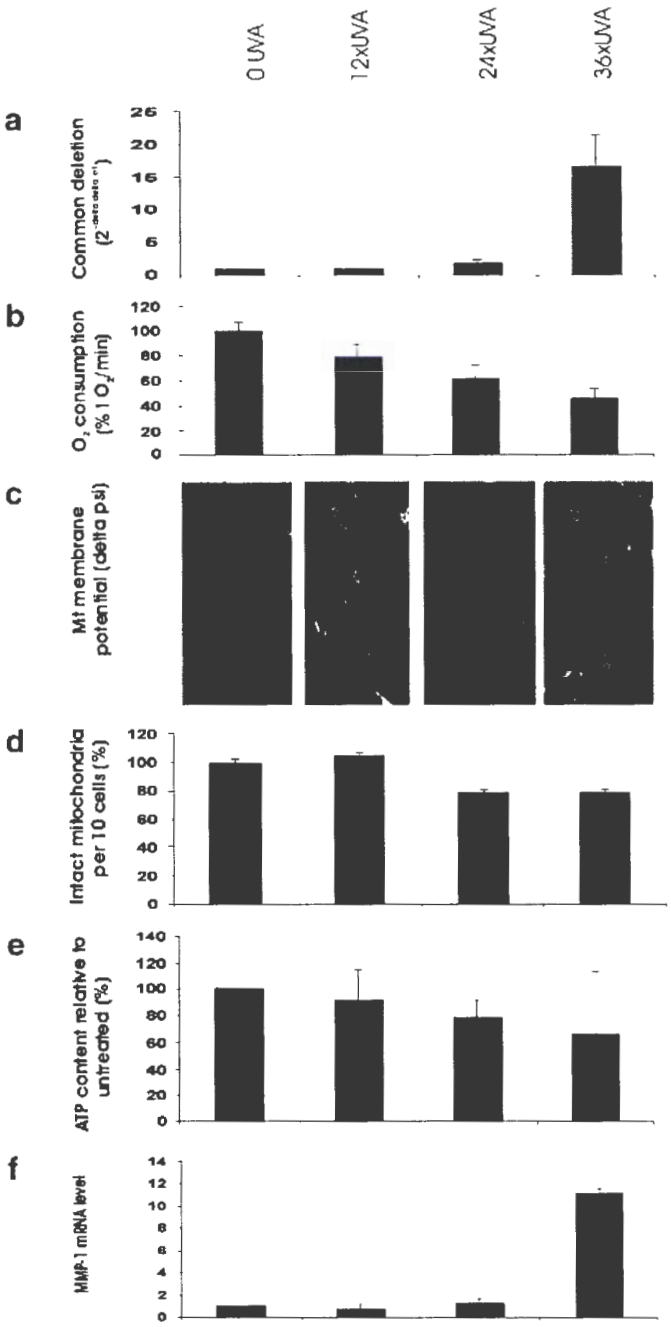

Figure 1
Induction of the common deletion in normal human fibroblasts after repetitive ultraviolet A light (UVA) irradiation leads to functional consequences. (*a*) Real-time PCR of common deletion is given as $2^{-\Delta\Delta Ct}$ of total existing mtDNA molecules in the examined sample with unirradiated samples set to 1, (*b*) measurement of oxygen consumption by Clark-type electrode in $\%IO_2$ per min with untreated cells set to 1, (*c*) fluorescence microscopic visualization of mitochondrial membrane potential. Red fluorescence indicates normal membrane potential, (*d*) quantification of intact mitochondria fluorescing red per 10 cells. Data are given as % of three microscopic fields with unirradiated cells set to 100%, (e) Cellular ATP content shown relative to untreated cells set as 100%,and (*f*) differential semiquantitative RT-PCR of matrix metalloproteinase mRNA levels in cells containing the common deletion with untreated cells set to 1. Normal human fibroblasts were irradiated as described in Materials and Methods. Positive control represents a sample of a patient with known disease caused by the common deletion. All lanes in *a–f* correspond to the top legend of 0, 12, 24, and 36 repetitive exposures to 8 J per cm^2 UVA. Data are given as mean ± SD of relative content of the common deletion of at least two experiments.

induction of the common deletion (Fig 1*c,d*). The reduction of $\Delta\psi$ in our system was continuous and not spontaneously reversible during the course of irradiation. Incubation of cells with carbonyl cyanide 4-(trifluoromethoxy)phenylbutazone (FCCP) at a concentration of 10 μM, known to completely depolarize the mitochondrial membrane potential, was able to reduce $\Delta\psi$ even further, indicating that the membrane potential was not completely abolished by UVA exposure (data not shown).

Reduction of ATP content As a first approximation of the cell's energy status, ATP levels were measured using a luciferase-based bioluminescence assay. Repetitively UVA-exposed cells showed continuously decreasing ATP levels similar to the reduction of oxygen consumption and $\Delta\psi$, with unirradiated cells exhibiting normal ATP contents (Fig 1*e*).

Expression of MMP-1 MMP have been described to play a pivotal role in a number of processes including carcinogenesis and photoaging. We performed differential RT-PCR for detection of mRNA levels of MMP-1, its tissue-specific inhibitor TIMP-1 and as housekeeping gene β-actin in cells containing the common deletion (Fig 1*f*). Levels of MMP-1 mRNA continuously increased during repetitive irradiation to a maximum after 3 wk, whereas there was no parallel upregulation of TIMP-1 or β-actin. Single exposure of cells to 8 J per cm^2 UVA did not lead to MMP-1 induction (data not shown).

The effect of creatine on mtDNA mutagenesis and functional parameters was assessed by measurement of the above parameters in the presence or absence of creatine.

No absorption of creatine in the UV-range In order to exclude the possibility that creatine does not act through restoration of the energy metabolism but merely through possible sun-protective properties, absorption of creatine in the UVA and UVB range was assessed. Creatine did not show any absorption at wavelengths ranging from 240 to 400 nm thus indicating that creatine does not absorb in the UVB or in the UVA range (Fig 2*a*).

No antioxidative capacity of creatine In order to exclude that biological effects of creatine are exerted through antioxidative capacity, lipid peroxidation of linoleic acid was measured photometrically with or without different concentrations of creatine ranging from 0.1 to 10 mM (Fig 2*b*). The positive control α-tocopherol delayed lipid peroxidation of linoleic acid up to 100 min while creatine did not show any delay of linoleic acid lipid peroxidation irrespective of the applied doses.

Uptake of creatine into cells In order to assess whether exposure of fibroblasts to creatine during repetitive irradiation leads to an increase of intracellular and intramitochondrial creatine levels, cells were incubated with radioactively labeled creatine. ^{14}C-creatine levels were measured autoradiographically in whole-cell extracts (WCE) after 24 h of exposure (Fig 2*c*) as well as WCE and mitochondrial extracts (Mito) after a complete course of irradiation (3 wk) (Fig 2*d*). There was a concentration-dependent increase of intracellular creatine concentration, with maximum values at 2 mM creatine (Fig 2*c*). When cells were repetitively irradiated in the absence of creatine, homogenates of whole-cell and mitochondrial extracts showed background levels of 20 counts per minute (CPM) per μg protein. In contrast to this, extracted homogenates of whole cells and mitochondria, previously treated with radioactively labeled creatine, showed a marked increase of creatine levels at values of 258 and 110 c.p.m. per μg protein, respectively (Fig 2*d*).

Effect of creatine on mtDNA mutagenesis and functional parameters Repetitive irradiation of fibroblasts in the presence of creatine (1 mM) completely abolished induction of the common deletion (Fig 2*e*). This effect could be slightly reduced when creatine was coincubated together with the competitive creatine uptake inhibitor arginine at 0.06%. To further assess the functional relevance of creatine-mediated protection from mitochondrial mutagenesis, the common deletion was measured, together with the mitochondrial membrane potential (Fig 2*e,f*) as well as oxygen consumption and MMP-1 mRNA levels (Fig 2*f*). Creatine incubation of cells in the absence of UV-irradiation led to supercompensation of the mitochondrial membrane potential at the creatine concentration used (Fig 2*e*, *middle and lower panel*). Furthermore, creatine coincubation improved cellular oxygen consumption as well as UVA-mediated upregulation of MMP-1 (Fig 2*f*).

Discussion

The data indicate that mtDNA deletions induced by repetitive UV-exposure at doses acquirable during a regular summer holiday can reach a critical level at which they are sufficient to impair mitochondrial function and to induce MMP-1. Furthermore, supplementation with the energy precursor, creatine, normalizes mitochondrial mutagenesis and functional changes through a mechanism independent from quenching effects of ROS.

It has been shown that phosphocreatine/creatine kinase exists in the human skin, with mitochondrial creatine kinase also being present (Schlattner *et al*, 2002), and that de novo synthesis of creatine has to be supplemented by food uptake. Furthermore, creatine has protective effects in neurodegenerative diseases and mitochondrial cytopathies (Baker and Tarnopolsky, 2003; Beal, 2003; Tarnopolsky *et al*, 2004). Therefore, together with the fact that ATP is insufficient as an external energy precursor due to its instability, to improve cellular energy metabolism, in our experiments, we used the commercially available energy precursor creatine.

Reduction of mitochondrial function UVA-mediated induction of the common deletion was paralleled by a decrease of mitochondrial function. The observed reduction of oxygen consumption in cells containing the common deletion in our experiments indicates a change in the metabolism of affected mitochondria with a magnitude large enough to be detected by measurement of oxygen consumption. This is confirmed by reduction of the mitochondrial membrane potential ($\Delta\psi$) as assessed by JC-1. Fluctuations of $\Delta\psi$ represent a normal state of mitochondrial

metabolism and are caused by gating of the mitochondrial permeability pore (Huser and Blatter, 1999). These fluctuations remain within physiological range, however, except during apoptosis. The fact that $\Delta\psi$ could be reduced further by coincubation with FCCP indicates that apoptotic levels of the mitochondrial membrane potential were not reached under experimental conditions.

In this study, decreased levels of ATP in cells containing the common deletion are evidence for a link between mutations of mtDNA and cellular energy metabolism. ATP and the cellular ATP/ADP ratio, however, are known to be unreliable parameters for cellular energy levels since they are maintained at homeostatic levels (Huser and Blatter, 1999; Baker and Tarnopolsky, 2003; Beal, 2003). Therefore, these data have to be considered as a general indication. Furthermore, ATP and phosphocreatine are unsuitable as experimental energy equivalents *in vitro* since they do not readily permeate the cell membrane due to their polarity. Therefore, since creatine has been effective in the restoration of symptoms in neuromuscular and mitochondrial diseases (Baker and Tarnopolsky, 2003; Beal, 2003), in order to provide further evidence for a role of energy metabolism in mtDNA mutagenesis, we investigated the effect of creatine on mtDNA mutagenesis and functional parameters. This implied the hypothesis that generation of phosphocreatine, and consequently ATP, is facilitated if creatine is abundant in cells. This would allow easier binding of existing energy-rich phosphates to the energy precursor creatine. Indeed, experimental supplementation of normal human fibroblasts with creatine normalized mitochondrial mutagenesis

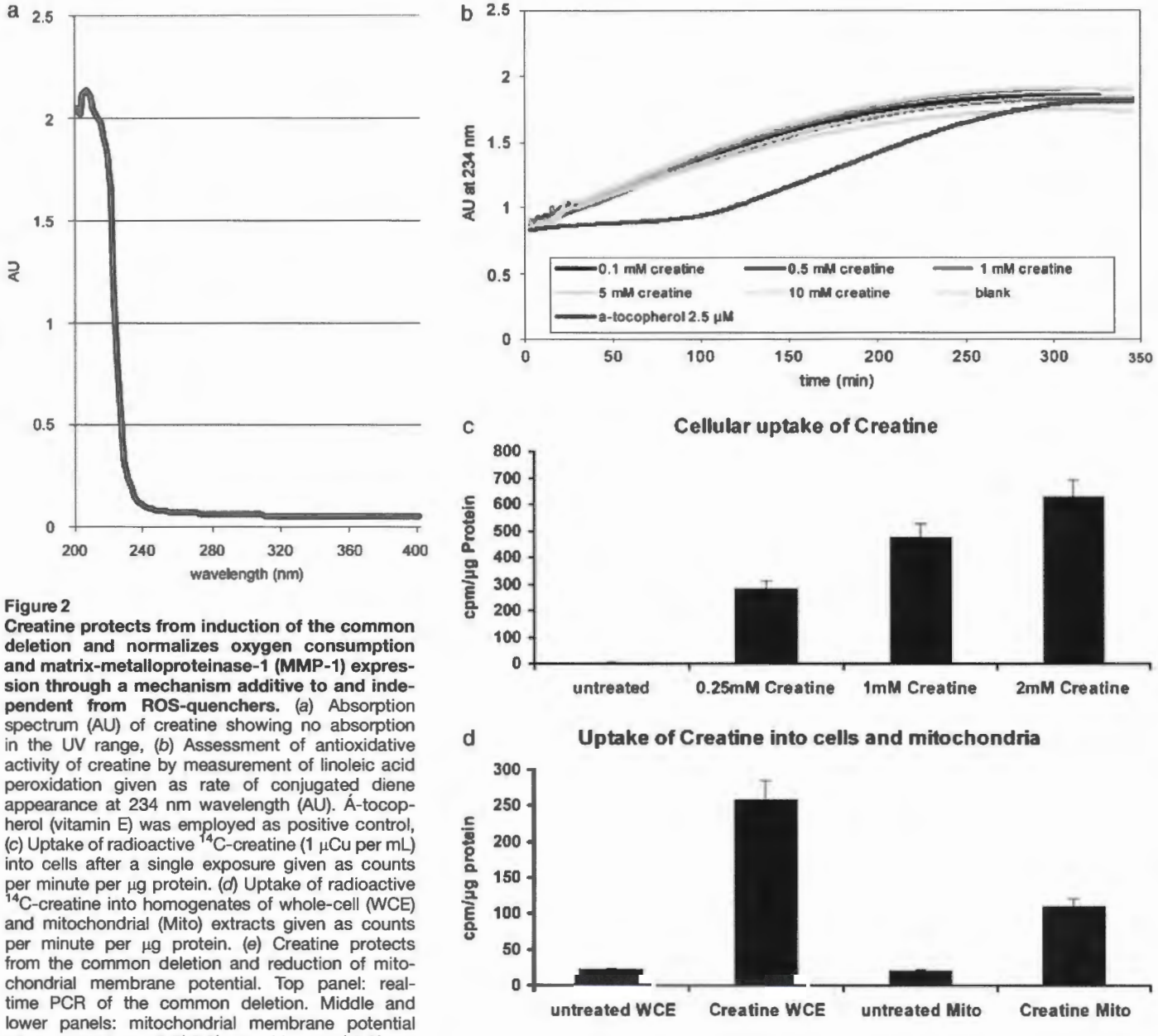

Figure 2
Creatine protects from induction of the common deletion and normalizes oxygen consumption and matrix-metalloproteinase-1 (MMP-1) expression through a mechanism additive to and independent from ROS-quenchers. (*a*) Absorption spectrum (AU) of creatine showing no absorption in the UV range, (*b*) Assessment of antioxidative activity of creatine by measurement of linoleic acid peroxidation given as rate of conjugated diene appearance at 234 nm wavelength (AU). Á-tocopherol (vitamin E) was employed as positive control, (*c*) Uptake of radioactive ^{14}C-creatine (1 µCu per mL) into cells after a single exposure given as counts per minute per µg protein. (*d*) Uptake of radioactive ^{14}C-creatine into homogenates of whole-cell (WCE) and mitochondrial (Mito) extracts given as counts per minute per µg protein. (*e*) Creatine protects from the common deletion and reduction of mitochondrial membrane potential. Top panel: real-time PCR of the common deletion. Middle and lower panels: mitochondrial membrane potential as assessed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1). (*f*) Protective effect of creatine from functional consequences. Top panel: real-time PCR of the common deletion. Second and third panels: mitochondrial membrane potential as assessed by JC-1. Third panel: oxygen consumption by Clark electrode. Bottom panel: MMP-1 mRNA levels detected by RT-PCR. Values are shown as in Fig and data are given as means ± SEM of at least two separate experiments.

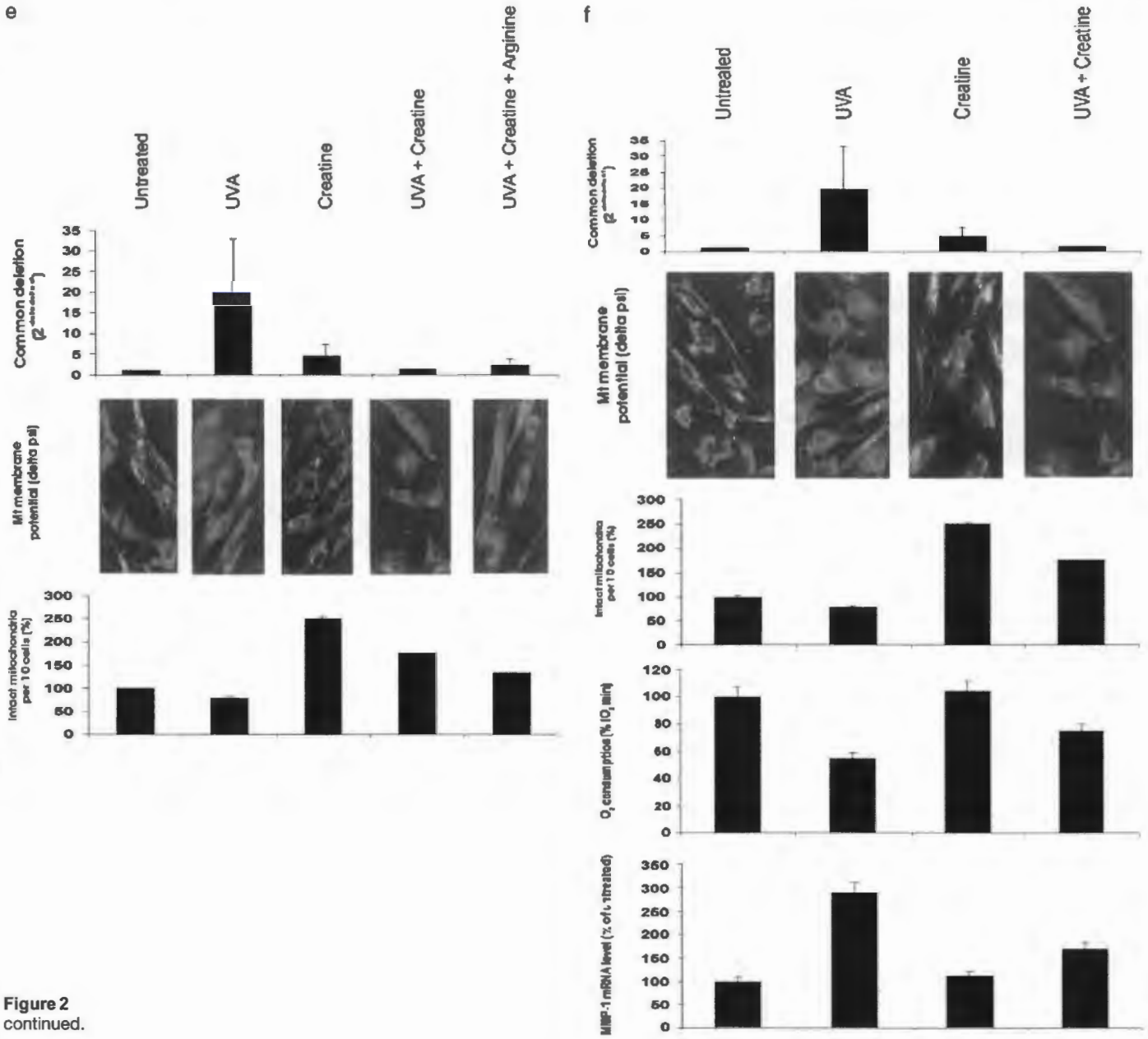

Figure 2
continued.

(Fig 2*e*), as well as the functional parameters oxygen consumption and MMP-1 (Fig 2*f*).

It has been shown that mutations of mtDNA are increased in chronically sun-exposed skin showing clinical signs of premature aging, also called photoaging (Yang *et al*, 1994, 1995; Berneburg *et al*, 1997, 1999, 2000, 2004; Birch-Machin *et al*, 1998), and it is known that photoaging is mediated through oxidatively-induced MMP-1 via the induction of macrophage inhibitory factor (MIF) in human dermal fibroblasts (Berneburg *et al*, 2000; Wenk *et al*, 2001; Watanabe *et al*, 2004). Furthermore, previous findings indicate that singlet oxygen is linked with the *in vivo* UVA action spectrum which is responsible for photoaging of mouse skin (Hanson and Simon, 1998). The fact that UV-induction of the common deletion is paralleled by an increase of MMP-1 whereas TIMP-1 remains unaltered, indicates a role of mtDNA mutations in the process of photoaging that is mediated by ROS-induced MMP-1.

The data demonstrate that creatine does not absorb in the UV range (Fig 2*a*) and does not act as an antioxidant (Fig 2*b*). Therefore, we hypothesize that protection from UVA-mediated induction of the common deletion is not exerted through direct quenching but through an indirect effect in which creatine, by normalizing the cell's energy status, reduces the requirement to upregulate a deleterious respiratory chain that again would generate more ROS.

Taken together, these data indicate that (i) repetitive, sublethal UV-exposure of cells may induce mtDNA mutations to a functionally relevant level and (ii) an increase of intracellular creatine levels is effective in protection from mtDNA mutations and the resulting functional consequences. Further experiments are necessary to clarify the precise interaction between induction of ROS, mitochondrial mutagenesis, and energy metabolism, but the antioxidant-independent effect of creatine may provide a tool to further

investigate underlying mechanisms of processes such as aging, photoaging, and carcinogenesis.

Materials and Methods

Cell culture Human normal skin fibroblasts were cultured in Eagle's minimum essential medium (PAA, Cölbe, Germany) containing 15% fetal calf serum (Perbio, Bonn, Germany). An osteosarcoma cell line lacking mtDNA (rho 0), serving as baseline control for measurement of oxygen consumption, was cultured in Dulbecco's minimum essential medium supplemented with 4.5 g per liter Glucose, 50 μg per mL uridine, and 5% fetal calf serum. Medium of all cells was supplemented with 1% L-Glutamine and 1% streptomycin/amphotericin B and they were kept in a humidified atmosphere containing 5% CO_2. Cells were kept in 10-cm culture dishes for culture and irradiation.

Mitochondrial extracts Mitochondria were isolated as described earlier (Wallace, 2001). All steps were carried out on ice. Briefly, 5×10^6 cells were trypsinized and pelleted with subsequent washing in mito-isolation buffer (comprising 210 mM mannitol, 70 mM sucrose, 1mM EGTA, 5 mM Hepes + 0.5% BSA). Pelleted cells were broken up by Digitonin (Sigma, Munich, Germany) and repeated mechanical treatment using a Teflon-homogenizer (Potters, Braun, Germany). Separation of mitochondria from cell debris was achieved by multiple wash steps with mito-isolation buffer, taking mt from the supernatant. Final centrifugation at 10,000 r.p.m. (12,823.46 *g*) for 20 min in mito-isolation buffer yielded the mitochondrial pellet.

Generation of the common deletion by UVA-irradiation Repetitive UVA-irradiation was performed as described previously (Berneburg *et al*, 1999; Eicker *et al*, 2003). In brief, for UVA-irradiation, medium was replaced by PBS, lids were removed, and cells were exposed to radiation from a UVASUN 5000 Biomed irradiation device (Mutzhas, Munich, Germany). The emission was filtered with UVACRYL (Mutzhas, Munich, Germany) and UG1 (Schott Glaswerke, Munich, Germany), and consisted of wavelengths greater than 340 nm. The UVA output was determined with a UVAMETER (Mutzhas, Munich, Germany) and was found to be approximately 70 mW per cm^2 UVA at a tube-to-target distance of 30 cm.

In order to generate the common deletion, cells were irradiated three times daily with 8 J per cm^2 UVA for four consecutive days and verified for viability by trypan blue exclusion. Cells were then aliquoted equally, with one aliquot stored at −80°C until extraction of mtDNA and another aliquot plated to a 10-cm culture dish for ongoing culture and irradiation. For assessment of functional endpoints, aliquots were processed as indicated below.

Chemical treatments All chemicals were purchased from Sigma and applied as described previously (Berneburg *et al*, 1999; Eicker *et al*, 2003) or as indicated. Vitamin E (α-tocopherol) was applied as described before (Berneburg *et al*, 1999; Eicker *et al*, 2003) at concentrations of 0.1 ng vitamin E per μL medium.

Mitochondrial mutagenesis

DNA extraction Extraction of total cellular DNA was carried out as described previously (Berneburg *et al*, 1997, 1999; Eicker *et al*, 2003; Berneburg *et al*, 2004).

Quantitative real-time PCR Experimental procedures for real-time PCR were carried out as described previously (Koch *et al*, 2001; Berneburg *et al*, 2004) using a TaqMan 7000 cycler (Applied Biosystems, Roth, Switzerland). In brief, amplification reactions were performed as 25 μL triplicates in a 96-well microplate format. Total mtDNA and deleted mtDNA reactions were conducted in separate tubes, each containing 100 ng DNA, 1 × Sybr green Master Mix, 500 nM of each IS primer, or 500 nM of each CD primer. Primer oligonucleotides and probes for the common deletion (CD) as well as the housekeeping gene (IS) identical to those published previously were used (Koch *et al*, 2001) and non-template controls showed undetectable fluorescence signals (ND). Values for the amount of common deletion are given as means in $2^{-\Delta\Delta Ct} \pm$ SEM.

Restriction enzyme analysis To confirm their identity, PCR products were subjected to diagnostic digestion with the restriction enzyme *Xba*I (New England Biolabs GmbH, Schwalbach, Germany).

Functional assessment

Oxygen consumption For measurement of oxygen consumption, cells were kept in DMEM (Gibco, Karlsruhe, Germany) medium containing no glucose supplemented with 1% of sodium-pyruvate, 1% penicillin/streptomycin and 1% glutamine, as well as 15% fetal calf serum. An aliquot of 3.5×10^6 cells was spun down at 1000 r.p.m. (128.23 *g*) for 5 min at room temperature, resuspended in DMEM, and kept on ice until measurement. The standard Clark type electrode (Hansatech, Kings Lynn, Great Britain) used was equilibrated according to the manufacturer's instructions with sodium-dithionite. Measurement of oxygen consumption was plotted in a time/oxygen-dependent manner, which was extrapolated by comparison with a standard curve to give values in liters oxygen consumption per minute.

Mitochondrial membrane potential ($\Delta\psi$) Assessment of mitochondrial membrane potential was performed by staining with the J-aggregate forming lipophilic cation JC-1 as provided by the ApoAlert mitochondrial membrane sensor kit (Clontech, Heidelberg, Germany). Measurements were performed as indicated in the kit. In brief staining stock solution was applied in tissue culture medium at 1 μL per mL medium and incubated in the dark at 37°C for 20 min. Fluorescence microscopy was performed using a Zeiss confocal laser microscope equipped with a 100-W mercury lamp (Zeiss, Oberkochen, Germany). For quantification, intact mitochondria fluorescing red were counted in at least three fields of vision for a total of 10 cells per microscopic field.

Content of ATP ATP determination in repetitively UVA-exposed cells was carried out by the ATP Bioluminescence Assay Kit HSII (Boehringer Mannheim, Germany). The HSII kit has been especially developed for the detection of ATP with highest sensitivity and uses ATP-dependent light emission by luciferase-catalyzed oxidation of luciferin. ATP measurement was performed according to the kit's protocol. In brief, cells were diluted to 10^5–10^8 cells per mL and automatically injected with 100 μL luciferase reagent and automatically measured in an MTP bioluminometer (Berthold, Bad Wildbach, Germany). Values were compared with standard solutions for the detection of ATP concentration.

RT-PCR for MMP-1, TIMP, and β-actin Detection of MMP-1, TIMP, and β-actin has been described previously (Watanabe *et al*, 2004). In brief, cells were harvested and total RNA was isolated, and mRNA expression was determined by semiquantitative differential RT-PCR (Stuart *et al*, 2004). For estimation of similar amounts of cDNA used for PCR, samples were screened for expression of β-actin as a "housekeeping" gene. The following primer oligonucleotides specific for MMP-1, TIMP, and β-actin, respectively were used: MMP-1: 5′-GTA TGC ACA GCT TTC CTC CAC TGC-3′, 5′-GAT GTC TGC TTG ACC CTC AGA GAC C-3′; TIMP: 5′-TTC CGA CCT CGT CAT CAG GG-3′, 5′-ATT CAG GCT ATC TGG GAC CGC-3′; and β-actin 5′-GTG GGG CGC CCC AGG CAC CA-3′, 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′. PCR products were separated electrophoretically in a 2% agarose gel, stained with the fluorescent dye Vistra green (Molecular Dynamics, Heidelberg, Germany) at a dilution of 1 in 10^5, and quantified fluorimetrically with a Storm 860 Phosphorimager (Molecular Dynamics).

Restoration of energy metabolism by creatine supplementation Cells were irradiated as described above in the presence or absence of 0.25, 1, and 2 mM creatine (Sigma). Creatine was

present in cell culture medium. It was removed and replaced prior to and after UV-exposure, respectively.

Absorbance spectrum of creatine The creatine absorbance spectrum was measured between 200 and 400 nm in a spectrophotometer (Spectra Max Plus, Molecular Devices, Sunnyvale, California), at a concentration of 10 mM in Dulbecco's phosphate-buffered saline 10 mM pH 7.4 (Invitrogen, Carlsbad, California).

No antioxidative capacity of creatine The antioxidative capacity of creatine was tested by the inhibition of linoleic acid peroxidation as modified from Liegeois *et al* (2000). Briefly, oxidation of linoleic acid was induced by using the radical initiator 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH). The progress of lipid peroxidation at 37°C was deduced from the rate of appearance of conjugated dienes at 234 nm, as measured in a spectrophotometer (Spectra Max Plus, Molecular Devices). Creatine was evaluated in a concentration range from 0.1 to 10 mM. α-Tocopherol (Sigma) was used as a positive control at a concentration of 2.5 μM.

Creatine uptake into cells and inhibition of uptake by arginine Radioactively labeled ^{14}C-creatine (Hartmann Analytics, Braunschweig, Germany) was added to fibroblasts following a wash with PBS. In the first set of experiments, the total cellular uptake of ^{14}C-creatine (1 μCu per mL) was assessed for differing concentrations of creatine ranging from 0.25 to 2 mM after 24 h of treatment (Fig 2*c*). In the second set of experiments, ^{14}C-creatine (1 μCu per mL) was coincubated together with non-radioactive creatine (2 mM) in order to avoid radioactive overload in culture medium three times daily for 3 wk along with the irradiation regimen. Arginine, a known competitor of creatine uptake, was applied as published previously (Walzel *et al*, 2002) at a concentration of 0.06% in culture medium. For both sets of experiments, incubation cells were washed with PBS and extracts were measured in a liquid scintillation counter (Beckmann coulter CS 6000 IC, Beckmann, Fullerton, California), and ^{14}C-counts were normalized on total protein amount (μg).

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Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury

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Nitrite has emerged as an endogenous signaling molecule with potential therapeutic implications for cardiovascular disease. Steady-state levels of nitrite are derived in part from dietary sources; therefore, we investigated the effects of dietary nitrite and nitrate supplementation and deficiency on NO homeostasis and on the severity of myocardial ischemia-reperfusion (MI/R) injury. Mice fed a standard diet with supplementation of nitrite (50 mg/liter) in their drinking water for 7 days exhibited significantly higher plasma levels of nitrite, exhibited significantly higher myocardial levels of nitrite, nitroso, and nitrosyl–heme, and displayed a 48% reduction in infarct size (Inf) after MI/R. Supplemental nitrate (1 g/liter) in the drinking water for 7 days also increased blood and tissue NO products and significantly reduced Inf. A time course of ischemia-reperfusion revealed that nitrite was consumed during the ischemic phase, with an increase in nitroso/nitrosyl products in the heart. Mice fed a diet deficient in nitrite and nitrate for 7 days exhibited significantly diminished plasma and heart levels of nitrite and NO metabolites and a 59% increase in Inf after MI/R. Supplementation of nitrite in the drinking water for 7 days reversed the effects of nitrite deficiency. These data demonstrate the significant influence of dietary nitrite and nitrate intake on the maintenance of steady-state tissue nitrite/nitroso levels and illustrate the consequences of nitrite deficiency on the pathophysiology of MI/R injury. Therefore, nitrite and nitrate may serve as essential nutrients for optimal cardiovascular health and may provide a treatment modality for cardiovascular disease.

dietary supplementation | myocardial infarction | nitric oxide

The loss of nitric oxide (NO) generation as a result of a dysfunctional vascular endothelium is an often cited correlate of heart disease (1). Continuous generation of NO is essential for the integrity of the cardiovascular system, and a decreased production and/or bioavailability of NO is central to the development of cardiovascular disorders (2, 3). NO is a highly reactive and diffusible gas formed by three NO synthase (NOS) isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). NO has been extensively studied in the setting of ischemia-reperfusion (I/R) injury. Previous studies clearly demonstrate that the deficiency of eNOS exacerbates myocardial I/R (MI/R) injury (4), whereas the overexpression of eNOS (5, 6), NO donor (7, 8), or inhaled NO gas (9) therapy significantly protect the myocardium (10). NO possesses a number of physiological properties that make it a potent cardioprotective-signaling molecule. These include vasodilation and the inhibition of oxidative stress, platelet aggregation, leukocyte chemotaxis, and apoptosis (11–13). NO synthesis is influenced critically by various cofactors, such as tetrahydrobiopterin, flavin mononucleotide, and flavin adenine dinucleotide, as well as the presence of reduced thiols, the endogenous NOS inhibitor asymmetric dimethylarginine, and substrate and oxygen availability. Without an adequate delivery of substrate and/or cofactors (i.e., ischemia), NOS function is compromised and NO production limited. Therefore, alternate means to produce NO in ischemic tissues is an attractive mechanism to limit I/R injury.

Nitrite is an oxidative breakdown product of NO that has been shown to serve as an acute marker of NO flux/formation (14). Nitrite recently has moved to the forefront of NO biology (15) because it represents a major storage form of NO in blood and tissues (16). In addition to the oxidation of NO, nitrite is also derived from reduction of salivary nitrate by commensal bacteria in the mouth and gastrointestinal tract (17, 18), as well as from dietary sources such as meat, vegetables, and drinking water. Much of the recent focus on nitrite physiology is attributable to its ability to be reduced to NO during ischemic or hypoxic events (16, 19–21). Nitrite reductase activity in mammalian tissues has been linked to the mitochondrial electron transport system (22), protonation (20), deoxyhemoglobin (23), and xanthine oxidase (24–25). Nitrite can also transiently form nitrosothiols (RSNOs) under both normoxic and hypoxic conditions (19), and a recent study by Bryan *et al.* (26) demonstrates that steady-state concentrations of tissue nitrite and nitroso are affected by changes in dietary NOx (nitrite and nitrate) intake. Previous studies have shown that nitrite therapy before reperfusion protects against hepatic and MI/R injury (25, 27). Additionally, experiments in primates have revealed a beneficial effect of long-term administration of nitrite on cerebral vasospasm (28). Oral nitrite has also been shown to reverse N^G-nitro-L-arginine methyl ester-induced hypertension and serve as an alternate source of NO *in vivo* (29).

A recent report by Kleinbongard *et al.* (30) demonstrates that plasma nitrite levels progressively decrease with increasing cardiovascular risk. Although a correlation exists in the plasma, it is not known whether the situation is mirrored in the heart and other tissues. If so, tissue nitrite may serve as an index of risk and restoring tissue nitrite may protect organs from ischemic injury. Because a substantial portion of steady-state nitrite concentrations in blood and tissue are derived from dietary sources (26), modulation of nitrite and/or nitrate intake may provide a first-line defense against ischemic heart disease. However, at present, there is no experimental evidence indicating the consequences of dietary nitrite or nitrate supplementation or deficiency on NO homeostasis or severity of I/R injury. We, therefore, investigated dietary nitrite and nitrate supplementation and insufficiency in mice, the effects of this manipulation on blood and heart nitrite/NO levels, and the effects of the manipulation on the severity of MI/R injury.

Results

Nitrite Supplementation Protects Against MI/R Injury by Increasing Plasma and Heart NO Stores. It is known that nitrite given intravenously immediately before reperfusion can protect from I/R injury

Author contributions: N.S.B., J.W.C., J.W.E., and D.J.L. designed research; N.S.B., J.W.C., J.W.E., S.G., and S.Y.J. performed research; N.S.B. and D.J.L. contributed new reagents/analytic tools; N.S.B., J.W.C., J.W.E., and S.G. analyzed data; and N.S.B., J.W.C., J.W.E., and D.J.L. wrote the paper.

Conflict of interest statement: D.J.L. is a participant in a pending U.S. patent, filed on October 14, 2003 through the National Institutes of Health (patent no. 60/511, 244), regarding the use of sodium nitrite in CVD. N.S.B. is on the scientific advisory board of TriVita, Inc.

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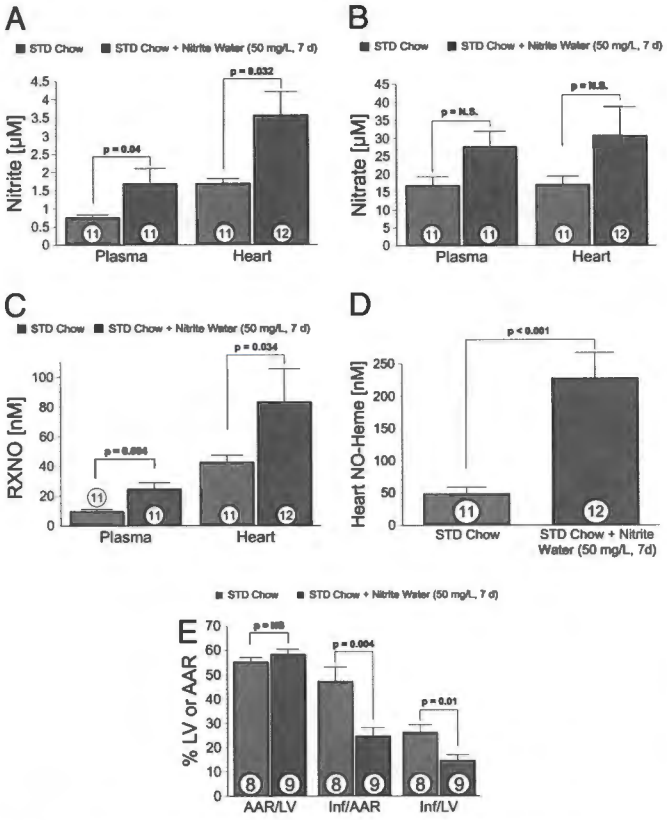

Fig. 1. Steady-state plasma and heart NOx and nitros(yl)ation levels. (*A–D*) Mice were fed a STD rodent chow with or without 50 mg/liter nitrite supplementation for 7 days, at which time steady-state levels of plasma and heart nitrite (*A*), nitrate (*B*), nitroso (RXNO) (*C*), and heart nitrosyl–heme (NO-Heme) (*D*) were measured. (*E*) Mice fed STD rodent chow supplemented with nitrite in their drinking water exhibited significantly higher plasma levels of nitrite, as well as significantly higher heart levels of nitrite, nitroso, and nitrosyl–heme. Nitrite supplementation significantly attenuated myocardial infarct size (Inf/AAR) by 44%. The numbers inside bars indicate the number of animals per group. LV, left ventricle.

(27). Because of the relatively short circulating half-life of nitrite in blood (110 seconds) (31), it is not clear whether nitrite administered subchronically in the drinking water can affect blood and tissue nitrite concentrations and therefore affect the outcome from an I/R insult. To test this notion, nitrite (50 mg/liter) was administered in the drinking water of mice on a standard (STD) rodent chow (Purina 5001; Purina) for 7 days. As shown in Fig. 1, there was a significant increase in steady-state concentrations of plasma nitrite (Fig. 1*A*) and nitroso products (RXNO) (*S*-nitrosothiols and *N*-nitrosoproteins) (Fig. 1*C*), as well as a significant increase in steady-state concentrations of heart nitrite (Fig. 1*A*), nitroso products (Fig. 1*C*), and nitrosyl–heme products (Fig. 1*D*). Plasma and heart nitrate (Fig. 2*B*) levels trended toward an increase, although no statistical significance was observed. To test whether nitrite supplementation and the biochemical changes resulted in protection against I/R injury, mice were subjected on the seventh day to 30 min of ischemia followed by 24 h of reperfusion. The area at risk (AAR) per left ventricle was similar for the mice maintained on a STD rodent diet (55.2 ± 1.8%) and those supplemented with nitrite (58.3 ± 2.1%) (Fig. 1*E*). The mice supplemented with nitrite water for 7 days displayed a 48% reduction in infarct size relative to the AAR (47.3 ± 5.8% vs. 24.7 ± 3.4%) and the infarct size relative to the entire left ventricle by 44% (26.1 ± 3.2% vs. 14.7 ± 2.3%). These data reveal that increasing nitrite dietary intake affects steady-state concentrations of cardiac nitrite, nitroso modified proteins, and nitrosyl–heme products and provides significant cardioprotection against I/R injury.

Nitrite Consumption During Myocardial Ischemia Leads to Increased Levels of Nitroso and NO–Heme Products in the Heart. Exogenous administration of nitrite has been shown to be protective in both the heart and the liver after I/R (25, 27). It has been speculated that nitrite is reduced to NO under ischemic conditions to provide an alternate source of NO when NOS is inactive because of decreased oxygen saturation and substrate delivery. Recent data also demonstrate that RSNOs are cytoprotective in I/R, possibly through NO-independent transnitrosation reactions (32). To better understand the fate of nitrite, we conducted a time course of nitrite metabolism after MI/R. As shown in Fig. 2, plasma nitrite (Fig. 2*A*) and nitroso (Fig. 2*C*, RXNO) are unchanged in control mice fed STD chow during I/R. Interestingly, plasma nitrate levels (Fig. 2*B*) increased by 44% ($P < 0.01$ vs. baseline) at the end of ischemia and remained at elevated levels for up to 30 min of reperfusion. The plasma nitrite (Fig. 2*A*) and nitroso (Fig. 2*C*) levels in the mice supplemented with nitrite water for 7 days were significantly higher than those levels measured in the mice fed a STD diet before the onset of ischemia. These levels remained higher after I/R. The plasma nitrite levels in these mice tended to decline (44% decrease by 30 min of reperfusion) during I/R, and there was a concomitant increase in plasma RXNO during the ischemic phase (Fig. 2*C*), which decayed during reperfusion.

The heart revealed a similar profile, but the changes were much more dramatic (Fig. 3). Mice on STD chow without nitrite supplementation revealed a trend for nitrite consumption during ischemia (Fig. 3*A*). Although the difference between the values at baseline and the end of ischemia was not significant, a 28% decrease in nitrite levels was observed. These levels remained decreased at 1 min of reperfusion and had almost rebounded to baseline values by 30 min of reperfusion. The mice supplemented with nitrite displayed significantly higher cardiac nitrite levels at baseline (Fig. 3*A*). The same trend was observed with nitrite levels, which decreased by 24% at the end of ischemia. However, the nitrite levels in these mice remained lower for up to 30 min of reperfusion, suggesting that nitrite is consumed during I/R. No significant changes in cardiac nitrate (Fig. 3*B*) were observed in either group at any time point evaluated. In sham-operated animals, the baseline levels of nitrite remained constant over a period of 60 min, suggesting that the nitrite levels were stable in our model system for at least 60 min (equivalent to 30 min ischemia plus 30 min of reperfusion). Furthermore, this observation suggests that the reduction of nitrite levels during ischemia is likely attributable to consumption and bioconversion to nitric oxide during ischemia. Along with a decrease in nitrite, there was a substantial and significant increase in both nitroso (Fig. 3*C*) and nitrosyl–heme products (Fig. 3*D*), both in the mice on a STD diet and the mice supplemented with nitrite, with slightly more in the supplemented group. During reperfusion, cardiac nitroso and nitrosyl–heme products decayed over time to reach near starting concentrations by 30 min of reperfusion.

Dietary Nitrite Deficiency Decreases Steady-State Levels of Nitrite and NO Metabolites and Exacerbates MI/R Injury. There is a growing appreciation that nitrite therapy may provide benefit from I/R injury (33). However, there are no data on the effects of nitrite insufficiency in the setting of I/R injury. To reveal the biochemical and physiological effects of dietary nitrite insufficiency, mice were fed a STD rodent chow (Purina 5001) for 9 wk and then switched to a purified amino acid diet low in nitrite and nitrate (TD 99366; Harlan) for 7 days. (Control mice were fed Purina 5001 for 10 wk) Consistent with an earlier report (26), the low NOx diet significantly decreased plasma and heart steady-state nitrite and nitrate concentrations, which could be restored by the addition of 50 mg/liter nitrite in the drinking water for 1 wk (Fig. 4 *A* and *B*). Blood and tissue nitroso products have been shown to preserve NO bioactivity

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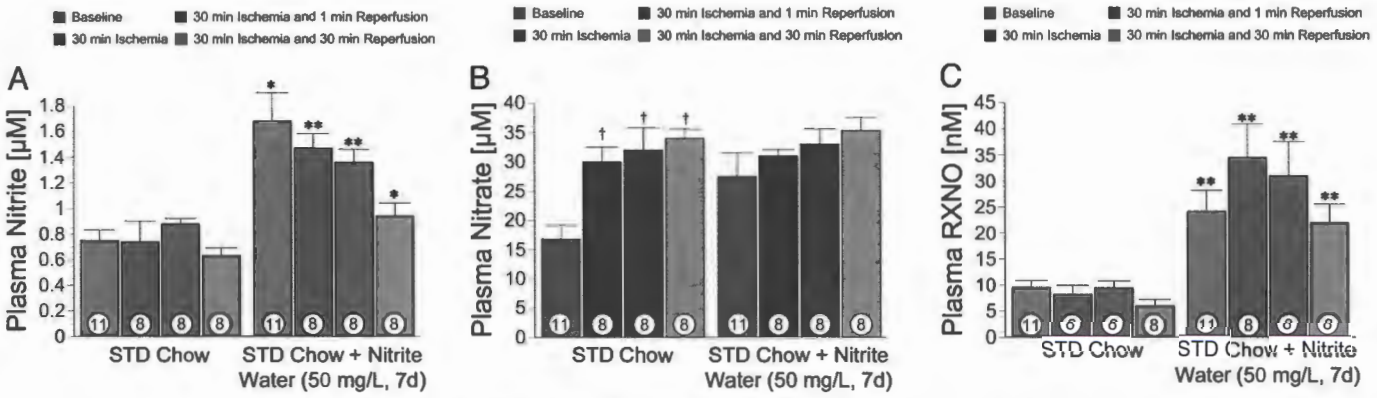

Fig. 2. Changes in plasma NOx and nitroso levels after MI/R. Mice fed a STD rodent chow with or without 50 mg/liter nitrite supplementation for 7 days were subjected to 30 min of left coronary artery ischemia followed by either 1 or 30 min of reperfusion. Plasma levels of nitrite (*A*), nitrate (*B*), and nitroso (*C*) were measured before ischemia (baseline), at the end of ischemia, at 1 min of reperfusion, and at 30 min of reperfusion. In the mice maintained on STD rodent chow, nitrite and nitroso levels remained unchanged during I/R, whereas nitrate levels increased during ischemia and remained at this elevated state during reperfusion. Mice supplemented with nitrite had higher baseline levels of nitrite, nitrate, and nitroso. The nitrite in these animals slowly declined over the course of I/R, whereas nitroso levels rose during ischemia and declined during reperfusion. Nitrate levels remained unchanged in these animals. *, $P < 0.05$; **, $P < 0.01$ vs. corresponding STD chow time point; †, $P < 0.01$ vs. STD chow baseline.

(34), and protein nitrosation modification confer cGMP-independent NO signaling events (35). We have previously shown that changes in dietary nitrite consumption affect cellular signaling events (26). Mice fed a low NOx diet for 1 wk demonstrated a significant reduction in plasma and heart nitroso levels compared with mice fed STD chow, which could be replenished and increased with 50 mg/liter nitrite in the drinking water for 1 wk (Fig. 4*C*). Nitrosyl–heme products (Fig. 4*D*) were also reduced in the mice fed a low NOx diet and replenished by nitrite supplementation in the drinking water. These data reveal that changes in dietary nitrite

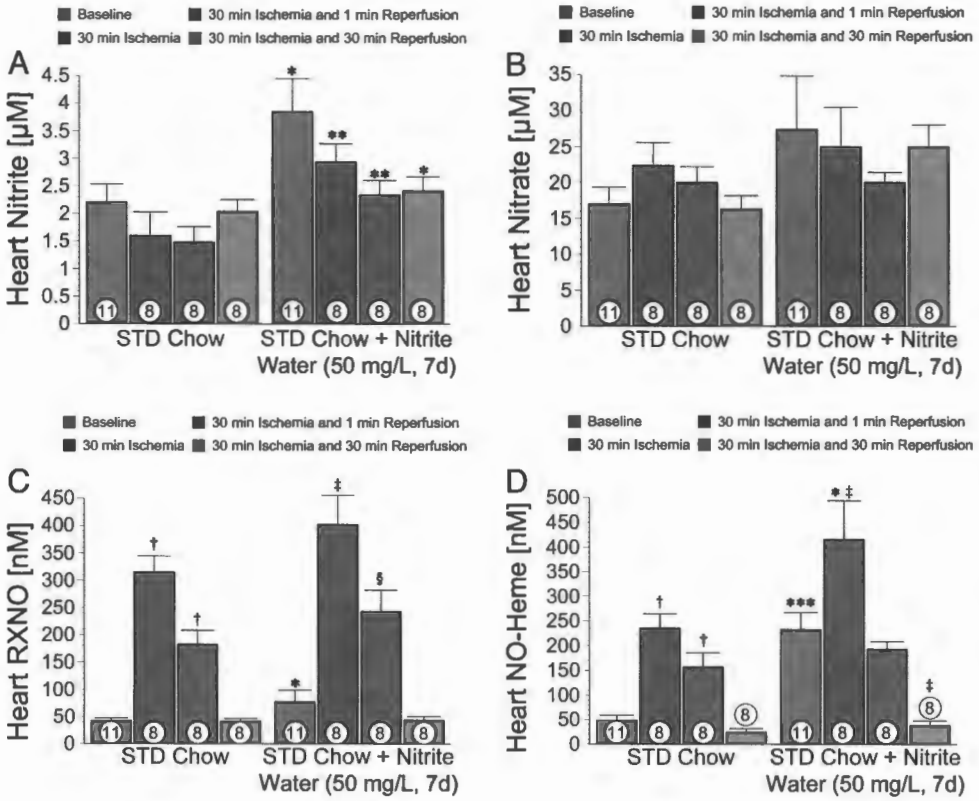

Fig. 3. Changes in heart NOx, nitroso, and nitrosyl–heme levels after MI/R. Mice fed a STD rodent chow with or without 50 mg/liter nitrite supplementation for 7 days were subjected to 30 min of left coronary artery ischemia followed by 1 or 30 min of reperfusion. Myocardial levels of nitrite (*A*), nitrate (*B*), nitroso (*C*), and nitrosyl–heme (*D*) were measured before ischemia (baseline), at the end of ischemia, at 1 min of reperfusion, and at 30 min of reperfusion. Mice supplemented with nitrite had higher baseline levels of nitrite, nitrate, nitroso, and nitrosyl–heme. Nitrite was consumed during the ischemic phase in both groups of mice with a concomitant increase in nitroso and nitrosyl products. During reperfusion, nitroso and nitrosyl products declined to near baseline levels by 30 min of reperfusion. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. corresponding STD chow time point; †, $P < 0.01$ vs. STD chow baseline; §, $P < 0.05$; ‡, $P < 0.01$ vs. STD chow plus nitrite water baseline.

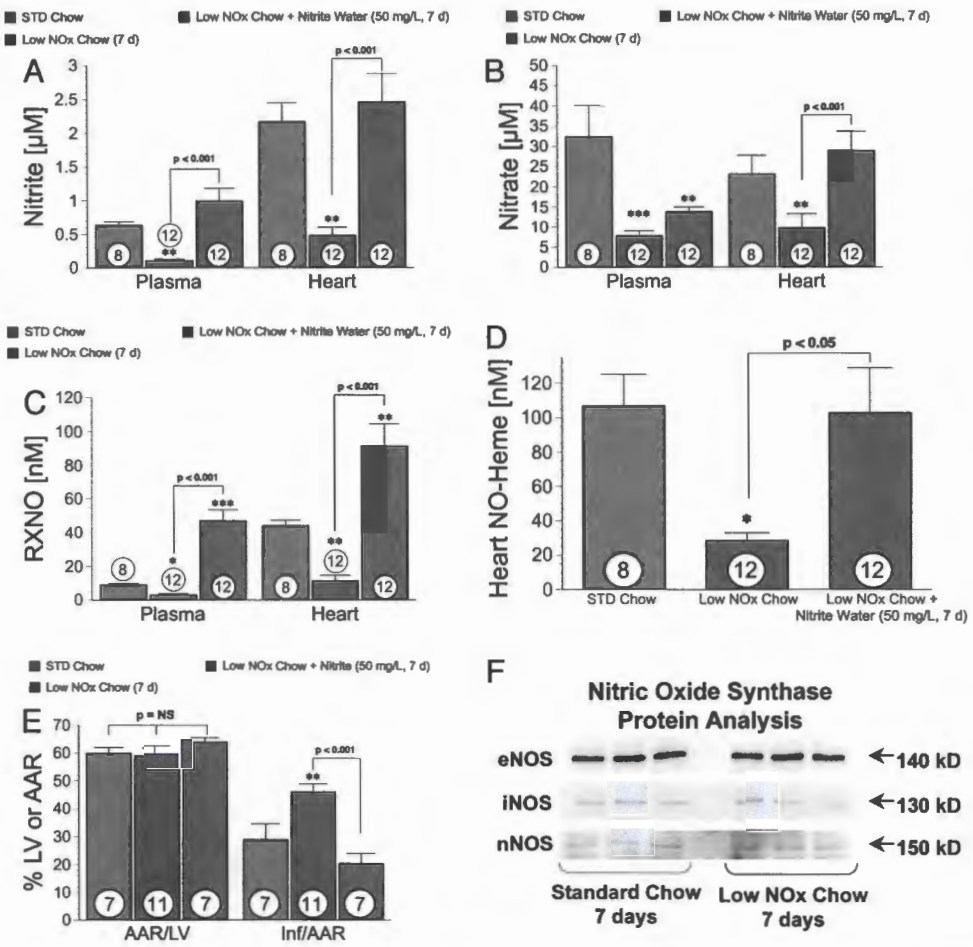

Fig. 4. Steady-state plasma and heart NOx and nitros(yl)ation levels in mice after nitrite insufficiency and supplementation. (*A–D*) Mice were fed a STD rodent chow or a low NOx chow with or without 50 mg/liter nitrite supplementation for 7 days, at which time steady-state levels of plasma and heart nitrite (*A*), nitrate (*B*), nitroso (*C*), and heart nitrosyl–heme (*D*) were measured. Consumption of low NOx chow resulted in a significant reduction in plasma and heart nitrite, nitrate, and nitroso, as well as heart nitrosyl–heme. The steady-state concentrations could be restored by supplementation of nitrite in the drinking water for 7 days. (*E*) Mice fed a low NOx diet for 7 days exhibited an exacerbated injury after MI/R. This increase in injury was reversed, however, in those animals fed a low NOx diet supplemented with nitrite. (*F*) Representative immunoblots of eNOS, iNOS, and nNOS from myocardial tissue homogenates of mice on STD chow and low NOx diet for 7 days. Animals on low NOx chow for 7 days did not show any changes in the protein expression of any of the isoforms of NOS. The numbers inside the bars indicate the number of animals per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. STD chow.

and/or nitrate consumption can affect steady-state concentrations of blood and tissue NO products/metabolites commonly used to assess NO production.

We next sought to determine whether dietary restriction of nitrite affected the severity of cardiac I/R injury. The decrease in steady-state nitrite concentrations in blood and heart was found to significantly exacerbate myocardial injury (Fig. 4*E*). The mice fed a low NOx diet displayed a 59% increase in myocardial infarct size relative to the AAR compared with mice fed a STD chow (29.0 ± 5.5% vs. 46 ± 2.8%). To ensure the observed effect depended on NOx intake, and not attributable to an alteration in the nutritional value of the low NOx diet, a subset of mice on the low NOx diet were given 50 mg/liter sodium nitrite ad libitum in the drinking water to restore steady-state concentrations of blood and tissue nitrite. Nitrite supplementation in animals on the low NOx diet reversed the previously observed increase in myocardial infarct size by 57% (46.0 ± 2.8 vs. 20.4 ± 3.6). Additionally, mice fed the low NOx diet displayed a higher mortality rate (58% survival) 24 h postmyocardial infarction than mice on the STD rodent chow (71% survival). Likewise, survival improved in mice on the low NOx diet with nitrite-supplemented drinking water to 77%. Because nitrite is derived both from diet and oxidation of enzymatic NO production from NOS, we investigated potential compensatory changes in NOS expression after 1 wk on low NOx diet. Western blot analysis of myocardial tissue lysate revealed no significant alterations in NOS expression (eNOS, nNOS, and iNOS) (Fig. 4*F*). These data suggest that the increased injury is attributable to changes in steady-state concentrations of plasma and heart nitrite as a result of decreased dietary NOx consumption and not from changes in enzymatic NO production.

Nitrate Supplementation Protects Against MI/R Injury by Increasing Steady-State Plasma and Heart Nitrite Levels. Because ≈25% of plasma nitrate is actively taken up by the salivary glands and secreted (36) and ≈20% of this nitrate is reduced to nitrite by commensal bacteria in the mouth (37–39), we investigated whether oral nitrate supplementation would increase steady-state plasma and tissue levels of nitrite. To test this notion, nitrate (1 g/liter) was administered in the drinking water of mice on a STD rodent chow (Purina 5001) for 7 days. As shown in Fig. 5, there was a significant increase in steady-state concentrations of plasma nitrite (Fig. 5*A*) and nitroso products (Fig. 5*C*), as well as a significant increase in steady-state concentrations of heart nitrite (Fig. 5*A*), nitroso products (Fig. 5*C*), and nitrosyl–heme products (Fig. 5*D*). Plasma nitrate levels increased in the nitrate-supplemented group (Fig. 5*B*). Heart nitrate levels trended toward an increase, although no

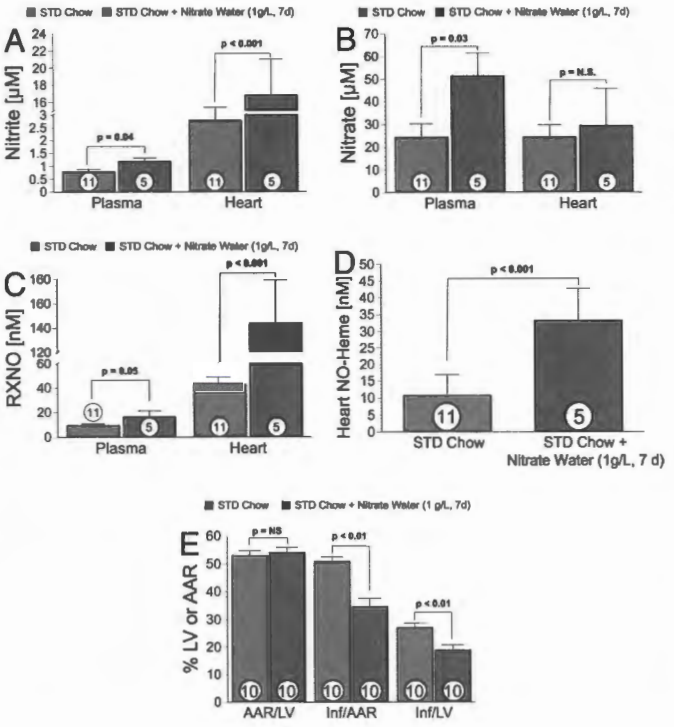

Fig. 5. Steady-state plasma and heart NOx and nitros(yl)ation levels. (*A–D*) Mice were fed a STD rodent chow with or without 1 g/liter nitrate supplementation for 7 days, at which time steady-state levels of plasma and heart nitrite (*A*), nitrate (*B*), nitroso (*C*), and heart nitrosyl–heme (*D*) were measured. (*E*) Mice fed STD rodent chow supplemented with nitrate (1 g/liter) in their drinking water exhibited significantly higher plasma levels of nitrite, as well as significantly higher heart levels of nitrite, nitroso, and nitrosyl–heme. Nitrate supplementation significantly attenuated myocardial infarct size (Inf/AAR) by 33%. The numbers inside the bars indicate the number of animals per group.

statistical significance was observed. The observed increases in plasma and heart nitrite and nitroso/nitrosyl levels afforded significant protection (Fig. 5*E*) against MI/R injury.

Discussion

The results of the present study demonstrate that modest changes in dietary nitrite and nitrate intake significantly alter steady-state concentrations of nitrite, nitroso modified proteins, and nitrosyl–heme products and that these biochemical changes have a profound outcome on the severity of acute myocardial infarction. It has long been appreciated that our diet exerts important long-term effects on vital body functions and impacts overall cardiovascular health and disease. There has been a well known connection between increased risk for cardiovascular disease (CVD) and poor diet, often involving high intake of saturated fat and limited fruit and vegetable intake, characteristic of a contemporary Western diet. However, dietary considerations usually focus on only fat and caloric intake with regard to preserving cardiovascular health but should now consider NOx intake as a dietary parameter for assessing cardiovascular risk. The data from this investigation reveal that mice are afforded significant cardioprotection from consuming 0.25 mg of nitrite per day and 5 mg of nitrate per day (assuming 5 ml of water consumption per day with 50 mg/liter nitrite and 1 g/liter nitrate). Interestingly, a report from the National Academy of Sciences (40) estimated, based on food consumption tables, that the average total nitrite and nitrate intake in the United States was 0.77 mg and 76 mg, respectively. Therefore, the cardioprotective levels reported in this current study can be achieved easily through increasing consumption of nitrite-/nitrate-rich foods. In fact, earlier reports by Lundberg and Govoni have revealed that high intake of nitrate results in increased systemic nitrite levels (38), and, most recently, it has been reported that dietary nitrate reduces blood pressure in healthy volunteers (41). The present findings suggest the possibility that nitrite-/nitrate-rich foods may provide protection against cardiovascular conditions characterized by ischemia. An optimal diet may then consist of a sufficient supply of nitrite or nitrate for health and protection from I/R injury. Regular intake of nitrite-containing food, such as green leafy vegetables, may ensure that blood and tissue levels of nitrite and NO pools are maintained at a level sufficient to compensate for any disturbances in endogenous NO synthesis (42). Because low levels of supplemental nitrite have been shown to enhance blood flow (23), dietary sources of NO metabolites could therefore improve circulation and oxygen delivery. This dietary pathway, therefore, may provide not only essential nutrients for NO production but also a rescue or protective pathway for people at risk for CVD (16). Moreover, any intervention that increases blood and tissue concentrations of nitrite may also provide protection against I/R injury. The paramount question then becomes whether some humans at risk for CVD are nitrite deficient? Indeed, a recent study suggests that plasma nitrite levels progressively decrease with increasing cardiovascular risk (30). Therefore, modest changes in the diet to include NOx rich foods may offer benefit and protection from those at risk for a myocardial infarction.

We have previously shown that steady-state RSNOs are linked to steady-state nitrite concentrations under normoxic conditions (26). Nitrite can also be reduced to NO under anaerobic conditions (21). Because both NO and RSNOs have now been shown to be protective in the setting of I/R (27, 32), nitrite now becomes a critical molecule in that it can form both NO and RSNOs. We propose that nitrite serves two functions in the setting of I/R. First, it serves as a NOS-independent source of NO by which nitrite is reduced to NO during ischemia when NOS is inactive. Second, nitrite reacts with critical thiols to form RSNOs. This nitroso modification may act as a reversible protective shield that prevents irreversible oxidation of proteins and lipids during the oxidative burst of reperfusion, or it may alter protein or enzymatic function and thereby modulate protective signaling pathways. Aside from thiol modification, we propose that the nitroso products can also release NO or the NO^+ moiety during the reperfusion phase and act as a redox-sensitive NO donor (43). Incidentally, the release of NO^+ will result in the instantaneous reaction of NO^+ with water to regenerate nitrite. Our biochemical data support this notion by the increase in nitroso at the expense of nitrite, followed by the decay of nitroso over time during reperfusion. Therefore, adding supplemental nitrite provides protection during I/R by not only increasing plasma and tissue levels of nitrite but also increasing steady-state levels of nitroso. On the contrary, nitrite insufficiency leads to increased injury because there are not enough nitrite or nitroso products stored in blood or tissue to perform these actions.

With one in every three men and one in every 10 women in the United States expected to develop CVD before reaching the age of 60 years (44), it is critical to examine preventive measures that promote cardiovascular health. NOx have generally been regarded as harmful substances because of their propensity to form *N*-nitrosamines, some of which are known carcinogens, but a causative link between nitrite or nitrate exposure and cancer is still missing (45). These data demonstrating protective effects of dietary NOx, along with numerous other reports suggesting the cardioprotective nature of dietary NOx (41, 42), warrant a paradigm shift on the nature of nitrite and nitrate in physiology and the food technologies industry. In summary, the findings of this current study demonstrate the influence of dietary NOx intake on plasma and myocardial levels of nitrite and NO and illustrate the cytoprotective effects of dietary NOx supplementation and consequences of NOx deficiency on the pathophysiology of MI/R injury. Furthermore, these data

suggest that dietary NOx may represent a means by which to attenuate MI/R injury.

Materials and Methods

Dietary Supplementation and Depletion of Tissue Nitrite. For the dietary supplementation studies, $NaNO_2$ or $NaNO_3$ was added to the drinking water of mice for 1 wk at concentrations of 50 mg/liter and 1 g/liter, respectively. For the nitrite depletion studies, mice were kept on the STD rodent diet (Purina 5001) and tap water for 9 wk before switching over to an amino acid diet (TD 99366; Harlan Teklad) with a matched L-arginine content and MilliQ water. The average NOx content of this diet was found to be considerably lower (20.5 ± 0.7 pmol/g nitrite and 503.1 ± 17.9 pmol/g nitrate) than that of the Purina rodent chow (104.5 ± 4.7 pmol/g nitrite and 6275 ± 50.7 pmol/g nitrate). There was no difference in daily chow consumption or weight gain between the groups. Nitrite supplementation was achieved by administering 50 mg/liter sodium nitrite in the drinking water to mice on the low NOx diet for 1 wk.

Tissue NO Products/Metabolite Determination. Biological specimens were harvested after 10 wk of STD diet or 9 wk of STD diet followed by 1 wk of low NOx diet for quantitative analyses of nitroso species and oxidation products of NO as detailed elsewhere (19, 46). No attempt was made to differentiate between a mercury-sensitive (RSNO) and mercury-insensitive (RNNO) adduct due to the limited blood and tissue volume from the mouse. NOx were quantified by ion chromatography (Eicom) (19).

Myocardial Infarction Protocol. *In vivo* ligation of the left coronary artery has been described in detail in refs. 5 and 27.

Myocardial Infarct Size Determination. Methods detailing Evan's blue perfusion to delineate ischemic vs. nonischemic areas and triphenyltetrazolium chloride staining for the calculation of myocardial infarct size have been described in detail in ref. 5.

Western Blot Analysis of NOS Isoforms. Western blot analysis was performed in a standard fashion as described in ref. 5. Myocardial lysate was transferred to PVDF membranes and incubated with the primary antibodies: mouse anti-eNOS, -iNOS, -nNOS, and -β-actin (loading control) in 5% BSA Tris-buffered saline with Tween 20 (TBST) overnight at 4°C. Membranes were then reacted with HRP-linked anti-mouse secondary at 1:2,000 in 5% BSA TBST, incubated with ECL reagents, and exposed to film.

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EFFECT OF A SHORT-TERM DIETARY CREATINE SUPPLEMENTATION ON HIGH-ENERGY PHOSPHATES IN THE RAT MYOCARDIUM

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The aim of this study was to find out whether creatine (Cr) feeding affects total creatine (TCr), phosphocreatine (PCr), adenine nucleotide contents and β-hydroxy-acyl-CoA-dehydrogenase (HAD) activity in myocardium as compared to red skeletal muscle. Ten adult Wistar rats received Cr (2.5 % of diet weight) for 7 days. In Cr fed rats, PCr was increased (by approx. 20%) in cardiac and in soleus muscles with ATP elevated in myocardium and TCr and free Cr in soleus. In both muscles, Cr feeding enhanced HAD activity. It is concluded, that dietary Cr does increase cardiac muscle high energy phosphate reserves and its oxidative potential.

K e y w o r d s: *creatine, phosphocreatine, ATP, β-hydroxy-acyl-CoA-dehydrogenase, rat myocardium, soleus muscle*

INTRODUCTION

Dietary creatine (Cr) supplementation for a few days was documented to improve performance of high-intensity exercise in men (1—3). This is attributed to increased ATP resynthesis in working muscles due to enhanced phosphocreatine (PCr) availability in type II fibers. Administration of Cr for 5 days was reported to increase resting PCr content both in type I and and type II fibers (by approx. 15%) in vastus lateralis muscle of healthy subjects (3). An increase in total Cr (TCr) and PCr content in skeletal muscles was also found in Cr fed rats (4—6). However, little is known on the effect of Cr supplementation on the heart. In patients with chronic heart failure Cr ingestion did not improve cardiac performance, evaluated on the basis of ejection fraction, although

exercise tolerance, TCr and PCr in skeletal muscle were enhanced (7). In the only experimental study on the effect of dietary Cr supplementation on the heart in animals, Horn *et al.* (8) failed to reveal any changes in the rat heart mechanical function, total Cr and PCr or ATP contents despite elevation in serum concentration of Cr by 73 to 202%, depending on the Cr food content.

The influence of muscle PCr content on oxidative potential of myocytes is still unclear. It was reported that depletion of PCr, by means of β-guanidinopropionic acid (GPA) administration, increases activities of mitochondrial enzymes in white (type IIb) skeletal muscle fibers but not in red fibers (type I) or in cardiac muscle (9). The data on the effect of dietary Cr supplementation on skeletal muscle oxidative enzymes are controversial (4,5), and these enzymes were not determined in the heart of Cr fed animals.

The aim of the present work was to investigate the effect of Cr supplementation on the contents of TCr, PCr, adenine nucleotides (ATP, ADP and AMP), as well as on the activity of β-hydroxy-acyl-CoA-dehydrogenase (HAD) in cardiac muscle of the rat. For comparison, the same variables were determined in the red skeletal muscle (soleus).

MATERIALS AND METHODS

The experiments were performed on 20 male Wistar rats, weighing 210 ± 5 g. All animals were housed in temperature controlled quarters (22°C) and had free access to drinking water. The rats were randomly assigned to one of two groups: Ten of them received for seven days 500 mg of monohydrate creatine daily (Now Foods, Glendale Hts., IL, USA) in 20 g of dry rat chow. Powdered chow diet was made to 2.5 % Cr, mixed into a paste with water, formed into pellets, and dried. The control group (10 rats) received daily 20 g of dry (similarly prepared) rat chow without Cr. On the 8th day rats were anesthetized with pentobarbital sodium (60 mg/kg body wt.). The soleus muscle and the apex part of heart ventricles were excised, deep frozen within 15 s in liquid nitrogen, and then stored at −80°C until assayed. The muscle specimens (about 50 mg of each) for determination of PCr, Cr, ATP, ADP and AMP were freeze-dried, dissected free of blood and connective tissue, powdered, and then extracted with perchloric acid. The neutralized extracts were analyzed enzymatically (10). Activity of HAD was determined in wet samples of soleus and cardiac muscles. The reactions catalyzed by this enzyme were coupled to NAD — NADP linked reactions according to Lowry and Passonneau (11).

Energy charge potential (ECP) was calculated according to Atkinson (12). All values are expressed as means with standard errors (SE). Statistical significance was assessed with unpaired Student's *t* test. Levels of significance were set at $P \leq 0.05$.

RESULTS

Creatine supplementation caused a significant increase of PCr both in cardiac and soleus muscles, by approx. 19% and 21%, respectively, whilst free Cr and TCr contents were enhanced significantly only in the soleus (*Table 1*).

In Cr fed rats, the cardiac muscle content of ATP was significantly enhanced, AMP was diminished, and ADP was unchanged. In the soleus muscle there was only a tendency towards an increase in ATP and a decrease in AMP in comparison with the control group. Neither in the heart nor in the soleus ECP was significantly affected by Cr supplementation.

Table 1. Total creatine (TCr), phosphocreatine (PCr), free creatine (Cr), adenine nucleotides (ATP, ADP, AMP) in $\mu mol \cdot g^{-1}$ d.w., energy charge potential (ECP), and PCr to ATP ratio in myocardium and soleus muscle of control (N) and creatine fed (C) rats.

		TCr	PCr	Cr	ATP	ADP	AMP	ECP	PCr/ATP
Cardiac muscle	N	90.7 ±2.6	38.5 ±2.1	52.2 ±3.2	23.3 ±1.1	4.1 ±0.8	0.37 ±0.06	0.91 ±0.02	1.67 ±0.10
	C	98.8 ±3.5	45.8* ±2.5	53.5 ±2.6	30.6* ±2.7	4.0 ±0.4	0.22* ±0.07	0.93 ±0.01	1.58 ±0.10
Soleus	N	102.3 ±2.0	57.0 ±2.3	45.2 ±3.3	23.9 ±1.3	4.6 ±0.4	0.52 ±0.12	0.91 ±0.12	2.46 ±0.18
	C	138.7*** ±3.4	9.2* ±5.3	69.5** ±7.2	26.0 ±2.0	4.6 ±0.5	0.32 ±0.05	0.92 ±0.01	2.73 ±0.21

Values are means ± SE. Asterisks denote significant differences between control and creatine fed rats: * $p<0.05$, ** $p<0.01$, *** $p<0.001$

In control rats, activity of HAD was nearly identical in myocardium and soleus muscle (20.2 ± 1.8 and 20.1 ± 1.3 $\mu mol \cdot min^{-1}\ g^{-1}$, respectively, and Cr feeding increased this enzyme activity to 34.2 ± 3.9 $\mu mol\ min^{-1}\ g^{-1}$ in the heart ($p<0.01$) and to 29.8 ± 2.8 $\mu mol\ min^{-1}\ g^{-1}$ in the soleus ($p<0.01$).

DISCUSSION

The present study showed that in the rat, seven day supplementation with Cr causes similar increases in PCr in cardiac muscle and in the red skeletal muscle, elevating significantly ATP content only in myocardium. This indicates that Cr feeding does enhance cardiac muscle energy reserves. The data did not, therefore, confirm the recent study by Horn *et al.* (8) who did not find any changes in the high energy phoshate content in myocardium of rats fed various doses of Cr (from 1 to 7% of a diet) for 40 days. The discrepancy between the results obtained by Horn *et al.* (8) and the present data may be related to duration of Cr feeding. It can be speculated that prolonged increase of extracellular Cr concentration may down-regulate the tissue creatine transporters (13, 14). There are also discrepancies in the literature concerning an influence of Cr supplementation on skeletal muscle PCr content. In the quoted

above study by Horn *et al.* (8) no effect of Cr feeding on skeletal muscle content of Cr and PCr was found, which is in contrast with the data obtained in human subjects (1, 2, 3) and in rats (4, 5, 6). It should be noted, that in the animal studies duration of Cr feeding and doses of Cr used as well as age and weight of animals varied. Op 't Eijnde *et al.* (6) who fed rats a high dose of Cr (5 mg g^{-1} body mass daily) for five days reported an increase in the soleus PCr content by 10 to 20%, which is similar to that found in the present study. Brannon *et al.* (5) followed up the time-course of changes in PCr and TCr contents in soleus and plantaris muscles up to 24 days after starting Cr feeding at a low dose (0.33% of diet). Their results showed that in both muscles PCr and TCr increased with the most pronounced changes occurring within the first 14 days. Both in the study by Horn *et al.* (8) and by Tanaka *et al.* (4) Cr was administered for a long time (40 and 54 days, respectively). However, in the latter study, in which a significant increase in muscle PCr was reported, Cr started to be given to newly weaned rats, while in the former investigation adult animals were used, and their body mass at the end of the experiment was above 400 g. It can be assumed, that ability to transport Cr to the muscle cells is greater in younger than in elder animals.

The important finding of the present study is that concurrently with the enhancement of high energy phosphate content induced by Cr supplementation there was an increase in HAD activity in both myocardium and soleus muscle. This is in line with the results of Brannon *et al.* (5) who reported an increase in citrate synthase activity in the soleus of Cr fed rats, thus suggesting, that in red skeletal muscles and myocardium an increase in CrP content increases mitochondrial oxidative potential. The data differ from those obtained by Tanaka *et al.* (4) demonstrating a decrease in HAD activity in the soleus muscle of rats after prolonged supplementation with Cr.

Both the human (15, 16) and animal studies (17) demonstrated that myocardial energy reserves are substantially reduced in chronic heart failure. Thus, the ability of dietary creatine to increase myocardial high energy phosphate content and oxidative potential may be of interest from the clinical point of view, although the role of energy flux by creatine kinase system in maintaining the contractile performance of the heart is still uncertain (17, 18).

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COMMISSION DIRECTIVE

on foods intended to meet the expenditure of intense muscular effort, especially for sports people

(Text with EEA relevance)

EXPLANATORY MEMORANDUM

1. Council Directive 89/398/EEC (OJ L 186, 30.6.1980, p. 27) of 3 May 1989 on the approximation of the laws of the Member States relating to foodstuffs intended for particular nutritional uses, as last amended by Directive 1999/41/EC (OJ L 172, 8.7.1999, p. 38) foresees the adoption by the Commission of a specific Directive on foodstuffs intended to meet the expenditure of intense muscular effort, especially for sportsmen. The Commission indicated in its White Paper on Food Safety that it would elaborate a specific Directive on foods intended to meet the needs resulting from intense muscular effort (Action No. 55).

2. On the basis of the opinion adopted by the Scientific Committee on Food (SCF) on 22 June 2000 (Report of the Scientific Committee on Food on composition and specification of food intended to meet the expenditure of intense muscular effort, especially for sportsmen. Adopted by the SCF on 22/6/2000, corrected by the SCF on 28/2/2001)) it is appropriate to lay down detailed compositional rules for the certain groups of foods with nutrient adaptation to meet the particular requirements associated with expenditure of intense muscular effort, especially for sports people.

3. The benefits of foods intended to satisfy the requirements associated with intense muscular effort can be useful not only for the sports people who are taking regular prolonged muscular exercise but also for other groups of the population, for example people in occupational jobs with hard physical work or extreme environmental conditions (people in the armed forces, miners, and mountaineers), as well as for people with irregular high intensity physical activities or fatiguing leisure activities.

4. The SCF note that a well-balanced diet is the basic nutritional requirement for athletes. However, aspects of physical activity (intensity, duration and frequency) and constraints of time mean there can be benefit from foods for particular nutritional purposes to satisfy the requirements associated with intense muscular effort. The increased energy needs of athletes are the most obvious difference in their nutritional requirements so their food intake is usually increased. This can affect food choice and eating patterns as well as having gastrointestinal effects.

5. Foods which give an increase in fluid, energy or nutrient availability in a short period of time can help to optimise performance directly or indirectly, through improved rate of recovery from intense muscular effort. Specially adapted nutritious foods or fluids which are easily digestible and rapidly absorbable may be useful to overcome the difficulty of providing high energy intakes when digestion and absorption can be impaired because of the intense muscular effort.

6. The metabolic capacity and power output of muscles depend on the energy source used. Maximal muscle performance depends almost entirely on carbohydrate as a substrate. People who take part in physical activity that involves intense muscular activity but who have low energy intakes need adequate carbohydrate intake to replace the carbohydrate used during the physical activity.

7. The scientific review of the SCF began in 1998. The report was published in 2000 and assessed the scientific evidence available at the end of 1990s relating to the particular nutritional requirements associated with activities involving intense muscular effort. At the time of the SCF review the following types of specific foodstuffs for sports people were on the market: rehydration drinks; energy drinks or energy powders and tablets; protein concentrates; supplements with specific vitamins, minerals and trace elements, and supplements with other substances such as creatine, choline and antioxidants; and, sports bars or meal replacement products.

8. On the basis of the available scientific evidence and the food products on the market at the time the SCF evaluated four groups of products intended to meet the particular nutritional requirements associated with the expenditure of intense muscular effort:

   - carbohydrate-rich energy food products;
   - carbohydrate-electrolyte solutions;
   - protein and protein components; and,
   - supplements – containing essential nutrients or other food components.

9. The SCF report indicates that the effect of high carbohydrate intake on physical performance has been extensively studied. The nutritional strategies to prepare for participation in and recovery from sport and exercise can be divided into pre-exercise meals, food intended to be consumed during exercise and food intended to be consumed after exercise. In terms of pre-exercise meals the method of "carbohydrate loading" during the week prior to competition is to gradually reduce the level of training during the week and to increase the carbohydrate intake to 9-10 grammes of carbohydrate per kilogramme bodyweight per day during the last days before the competition. It is possible to eat during participation in only a few sports. Easy to digest carbohydrate snacks or carbohydrate-electrolyte solutions are often eaten during such events. Regarding nutrition to aid recovery following exercise the SCF noted that research showed that a high carbohydrate diet during the post exercise period restored endurance performance during subsequent exercise. Therefore, carbohydrate-rich energy food products are useful when an athlete has a limited period of time for recovery between periods of prolonged physical activity.

10. With respect to carbohydrate-electrolyte solutions the SCF explain that the two factors that have been considered to contribute most to the onset of fatigue in exercise are the depletion of the body's carbohydrate reserve and the onset of dehydration. The SCF report indicates that compared to water during prolonged physical activity drinks containing carbohydrates and electrolytes improve performance. However, the optimum carbohydrate concentration depends on a number of factors, among others the need for water and the intensity and type of exercise, intestinal absorptive capacity, osmolarity and type of carbohydrates. High carbohydrate concentrations delay gastric emptying, reducing the amount of fluid that is available for absorption but increasing the rate of carbohydrate delivery. The SCF advised an energy range of 80-350 kilocalories per litre for carbohydrate-electrolyte solutions with at least 75 % of energy provided by carbohydrate. The SCF noted that the addition of sodium stimulates carbohydrate and water uptake and helps to

maintain extracellular fluid volume and they recommended a minimum level of sodium in such products.

11. The SCF noted that endurance athletes have a modest increase in protein requirements. The Committee considered that a diet containing 10-11 % energy from protein would meet the protein requirements of athletes who have an increased energy requirement. The SCF noted, however, that the increased requirement for protein might not be met if the total energy intake is relatively low. The SCF made recommendations for the protein content of protein concentrates and protein enriched foods.

12. The SCF noted that with an adequate dietary intake there is no need for additional essential micronutrients. Although in the case of restricted food intake, as seen in weight related sports, micronutrient intake could become marginal or deficient and under certain circumstances some athletes may not be able to reach their daily micronutrient requirements during periods of regular training. The SCF conclude that scientific evidence was lacking or inconsistent in supporting recommendations for nutrient intakes beyond population reference intakes.

13. The SCF reviewed a number of food components that had been related to physical performance; caffeine, creatine, carnitine, medium-chain triglycerides and branch chain amino acids. The SCF considered that there was scientific data of an ergogenic effect for only caffeine and creatine.

14. In a separate opinion adopted on 7 September 2000 the SCF reviewed the safety aspects of creatine supplementation (Opinion of the Scientific Committee on Food on safety aspects of creatine supplementation. (Adopted on 7 September 2000)). The Committee noted that certain intakes of creatine are effective in increasing total muscle creatine and improving performance of short term high intensity exercise. The SCF recommended that high loading doses of creatine should be avoided. On the basis of this opinion it is appropriate that products containing added creatine should have detailed instructions for use.

15. The proposed rules would contribute to a high level of protection of consumer interests by ensuring that the foods marketed as satisfying the particular nutritional requirements associated with expenditure of intense muscular effort have an appropriate composition, are safe and labelled in an adequate and clear manner, allowing consumers to make informed choices.

Note

The World Anti-Doping Agency (WADA) was established in 1999. Its mission is to promote and co-ordinate at international level the fight against doping in sport in all forms. WADA co-operates with intergovernmental organisations, governments, public authorities and other public and private bodies fighting against doping in sport. WADA is entitled to make proposals to the Olympic Movement, to international sports organisations and to public authorities on measures that could be taken to ensure further harmonisation and equity in anti-doping questions.

In 2003 WADA published the World Anti-doping Code (World Anti-Doping Agency, The World Anti-Doping Code, March 2003). The Code refers to the "Prohibited list" which includes a list of prohibited classes of substances whose use is regulated or proscribed in sport, in particular sports associated with the Olympic Movement. The list is published in

January each year and updated as necessary, the last updated list was published on 17 March 2004 and it came into effect on 26 March 2004.

The Commission considers that foods intended to satisfy the specific nutritional requirements associated with intense muscular effort, especially for sports people, should not contain prohibited substances included in the WADA prohibited list. However, the way to effectively ban such substances by a Commission Directive is not obvious. The compilation of such a list by the Commission is not considered possible at this stage. Whilst it is not possible to introduce in EU legislation a prohibition on the addition of substances on a list which is not under EU legislative control.

Member States are invited to consider this issue and views on the possible ways forward would be welcome.

Draft

COMMISSION DIRECTIVE

on foods intended to meet the expenditure of intense muscular effort, especially for sports people

(Text with EEA relevance)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 89/398/EEC of 3 May 1989 on the approximation of the laws of the Member States relating to foodstuffs intended for particular nutritional uses and in particular Article 4 (1) thereof, (OJ L 186, 30.6.1989, p. 27, as last amended by Directive 1999/41/EC (OJ L172, 8.7.1999, p38).)

After consulting the European Food Safety Authority,

Whereas:

(1) Foods intended to meet the expenditure of intense muscular effort should meet the particular nutritional requirements of people who participate in activities that involve intense muscular effort, in particular professional sports people but also other people for example those in the armed forces, mountaineers, and manual workers such as miners. Such products may also be used by individuals who participate in activities that involve intense muscular expenditure during their leisure time.

(2) The foods covered by this Directive are intended to be consumed in addition to or as a partial substitute for a normal diet.

(3) The opinions adopted by the scientific advisory body concern foods intended to satisfy the particular nutritional requirements associated with intense muscular activity, namely carbohydrate-rich energy foods, carbohydrate-electolyte solutions, protein concentrates and protein enriched foods. (Report of the Scientific Committee on Food on composition and specification of food intended to meet the expenditure of intense muscular effort, especially for sportsmen. Adopted by the SCF on 22/6/2000, corrected by the SCF on 28/2/2001 and the Opinion of the Scientific Committee on Food on safety aspects of creatine supplementation. (Adopted on 7 September 2000).)

(4) Given the nature of these products it is appropriate to lay down detailed compositional rules for foods with nutrient adaptation to meet the particular requirements associated with expenditure of intense muscular effort, especially for sports people.

(5) The scientific advisory body noted that certain levels of intakes of creatine could have an impact on certain types of exercise, however, it considered that high

loading doses of creatine should be avoided. It is appropriate that labelling requirements for food products containing added creatine covered by this Directive should be introduced.

(6) Pursuant to Article 7 of Directive 89/398/EEC, the products covered by that Directive are subject to the general rules laid down in Directive 2000/13/EC of the European Parliament and of the Council on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs. The present Directive adopts and expands upon the additions and exceptions to those general rules, where appropriate. (OJ L 109, 6.5.2000, P. 29. as last amended by Commission Directive 2001/101/EC (OJ L 31, 28.11.2001, p19).)

(7) In particular, in view of the nature and destination of foods intended to meet the expenditure of intense muscular effort it is necessary to provide information concerning the energy value and principal nutrients contained in such foods and, where appropriate, the origin and nature of the protein and/or protein hydrolysates and the osmolality or osmolarity.

(8) Electrolyte solutions that are "isotonic" have specific characteristics, therefore, it is appropriate to specify certain criteria for the use of the term if it is included in the labelling of these products.

(9) In accordance with the principle of proportionality, it is necessary and appropriate for the achievement of the basic objective of approximating the laws of the Member States relating to foodstuffs intended for particular nutritional uses to lay down rules on foods intended to meet the expenditure of intense muscular effort, especially for sports people. This Directive does not go beyond what is necessary in order to achieve the objectives pursued in accordance with the third paragraph of Article 5 of the Treaty.

(10) The measures provided for in this Directive are in accordance with the opinion of the Standing Committee on the Food Chain and Animal Health,

HAS ADOPTED THIS DIRECTIVE:

Article 1

1. This Directive is a specific Directive within the meaning of Article 4 (1) of Directive 89/398/EEC and lays down compositional and labelling requirements for foods intended to meet the expenditure of intense muscular effort as defined in Article 2 (1) and presented as such.

Article 2

1. For the purpose of this Directive, the following definition shall apply:

"foods intended to meet the expenditure of intense muscular effort" means a category of foods for particular nutritional uses specially processed or formulated and intended to meet the nutritional requirements for the expenditure of intense muscular effort, including but not limited to requirements associated with sporting activities.

2. Foods intended to meet the expenditure of intense muscular effort are classified in the following four categories: See note 1

(a) carbohydrate-rich energy food products with a specific nutrient adapted formulation to meet the particular energy requirements associated with the expenditure of intense muscular effort;

(b) carbohydrate-electrolyte solutions with a specific nutrient adapted formulation to meet the particular energy and electrolyte requirements associated with maintaining

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Note 1 – The SCF evaluated the scientific evidence on the nutritional requirements for the expenditure of intense muscular effort and considered the specific food products that existed on the market at the end of the 1990s. The SCF recommended specific compositional requirements for 4 categories of foodstuffs. The SCF noted that the scientific evidence supported an ergogenic effect of caffeine and creatine but no specific compositional requirements were specified in their report for such products.

During the last few years there have been further developments in the area of products intended for use before, during and after sporting activities. For example there are now drinks intended for sports people specifically designed for hydration but with an energy content of less than 80 kcal/l. In addition, interested parties have asked for clarification of the situation of other product categories such as supplements, weight gain or body building powders and meal replacements with respect to the Directive.

The additional categories of products that need to be considered are not well defined. There are two options to include other categories of products in the scope of the Directive:

a) With the involvement of stakeholders the different categories that exist on the market at the present time could be described. The European Food Safety Authority could then be asked to give a scientific opinion on the specific compositional requirements for the different categories that have been identified.

b) The Directive could include a category of products that would be open and that would allow existing products and new products to be marketed subject to a notification procedure. When placing a product on the market the manufacturer or importer of a product would notify the competent authority of the Member State by forwarding a model of the label used for the product. The manufacturer or importer would be expected to keep a file supporting the marketing of the product for the intended uses as they could be required to produce for the competent authority the scientific work and the data establishing the product's compliance with the requirements of Article 1 (2) of Council Directive 89/398/EEC.

hydration before and during the expenditure of intense muscular effort or restoring hydration after the expenditure of intense muscular effort;

(c) protein concentrates with a specific nutrient adapted formulation to meet the particular nutritional requirements for protein associated with the expenditure of intense muscular effort;

(d) protein enriched foods with a specific nutrient adapted formulation to meet the particular nutritional requirements for protein associated with the expenditure of intense muscular effort;

Article 3

Member States shall ensure that the products referred to in paragraphs 1 and 2 of Article 2 may be marketed within the Community only if they conform to the definition and rules laid down in this Directive.

Article 4

1. The formulation of foods intended to meet the expenditure of intense muscular effort shall be based on sound nutritional principles. Their use, in accordance with the manufacturer's instructions, shall be safe and beneficial and effective in meeting the particular nutritional requirements of the persons for whom they are intended, as demonstrated by generally accepted scientific data.

2. Products referred to in points (a) to (d) of Article 2 (2) shall comply with the compositional criteria specified in the Annex.

Article 5

1. The name under which products covered by this Directive are sold shall be "dietary food for physical activity" or, if appropriate, "dietary drink for physical activity". If the product is intended to satisfy the nutritional requirements associated with a specific type of physical activity then the type of physical activity may be also included in the name of the product. Where the physical activity is associated with a sport then the name of the sport for which the product is intended may be indicated in association with the name of the product.

2. The labelling shall bear, in addition to those provided for in Article 3 of Directive 2000/13/EC, the following mandatory particulars:

a) the available energy value expressed in kJ and kcal, and the content of protein, carbohydrate and fat, expressed in numerical form, per 100 g or per 100 ml of the product as sold and where appropriate per 100 g or 100 ml of the product ready for use in accordance with manufacturer's instructions. This information may in addition be provided per serving as quantified in the label or per portion, provided that the number of portions contained in the package is stated;

b) for products specified in points (c) and (d) of Article 2 (2) information on the origin and the nature of the protein and/or protein hydrolysates contained in the product;

c) for creatine and products with added creatine detailed instructions for use shall be provided. Such instructions shall result in intakes of not more than 3 g [See note 2] creatine per day;

d) where appropriate, information on the osmolality or the osmolarity of the product.

3. The following description may be included in the labelling of drinks according to their osmolarity:

- drinks with an osmolality not less than 270 mOsm/kg water and not greater than 330 mOsml/kg water may be described as "isotonic" or an equivalent description. [See note 3]

4. The labelling shall bear instructions for the appropriate preparation, the use and the storage of the product after opening of the container, as appropriate.

Article 6

1. Member States shall permit trade in products which comply with this Directive from [...] at the latest.

Note 2 – Interested parties have requested that the maximum permitted level of regular creatine supplementation be 5g/day with the possibility for higher levels during periods of creatine loading.

2. Member States shall prohibit trade in products which do not comply with this Directive from [...] at the latest.

Article 7

1. Member States shall bring into force the laws, regulations and administrative provisions necessary to comply with this Directive by [...] at the latest. They shall forthwith communicate to the Commission the text of those provisions and a correlation table between those provisions and this Directive.

When Member States adopt those provisions, they shall contain a reference to this Directive or be accompanied by such a reference on the occasion of their official publication. Member States shall determine how such reference is to be made.

2. Member States shall communicate to the Commission the text of the main provisions of national law which they adopt in the field covered by this Directive.

Article 8

This Directive shall enter into force on the 20th day following that of its publication in the *Official Journal of the European Union*.

Article 9

This Directive is addressed to the Member States.

Done at Brussels, [...]

For the Commission
[...]
Member of the Commission

Note [3] – The SCF report proposed the term "isotonic" may be applied to products with an osmolality of 270 – 330 mOsml/kg water. The need to define criteria of terms for drinks with other ranges of osmolalities may need to be considered.

ANNEX

ESSENTIAL COMPOSITION OF FOODS INTENDED TO MEET THE EXPENDITURE OF INTENSE MUSCULAR EFFORT

Note - The specifications refer to the products ready for use, marketed as such or reconstituted as instructed by the manufacturer.

CARBOHYDRATE-RICH ENERGY FOOD PRODUCTS

1. Products referred to in point (a) of Article 2 shall contain:

Carbohydrate: Carbohydrates shall provide at least 75 % of total energy See note 4

> in the case of drinks the product must have a carbohydrate concentration of at least 10 % of weight by volume See note 5 and, metabolisable carbohydrates See note 6 shall provide at least 75 % of total energy.

If vitamin B_1 (thiamin) is added, the product shall contain at least 0.2 mg vitamin B_1 per 100 g carbohydrates.

Note 4 – Interested parties have suggested that the minimum energy from carbohydrate should be 60 % or 65 %. They indicate that in addition to carbohydrate the raw materials used in the manufacture of solid food products contain protein and lipids that make it difficult to meet the minimum threshold of 75 % energy form carbohydrate. They also suggest that this threshold will reduce the range of acceptable, palatable products for sports people.

Note 5 – Interested parties have suggested that the minimum level of carbohydrate in carbohydrate-rich energy drinks should be greater than 10 % weight by volume. They suggest that a higher level of carbohydrate would enable the drink to provide more energy rather than optimal fluid replacement. They suggest that the minimum carbohydrate content should be 15 % weight by volume or should be at least 50% greater than standard soft drinks and juices which contain 10-12 % weight by volume carbohydrate.

Note 6 – The SCF proposed that the carbohydrate source should be metabolisable carbohydrate characterised by a high glycaemic index and gave as examples glucose, glucose polymers and sucrose. It should be noted that the definition of a "high" glycaemic index carbohydrates and the control of it in food products, if it were to be specified in a legal provision, would pose numerous problems. In addition, the characterisation of carbohydrates by high glycaemic index excludes fructose and interested parties have indicated fructose has been shown to be useful in foods intended for sports people.

CARBOHYDRATE-ELECTROLYTE SOLUTIONS See note 7

2. Products referred to in point (b) of Article 2 shall contain:

Energy: The energy content shall be at least 340 kJ/l (80 kcal/l) See note 8 and not greater than 1488 kJ/l (350 kcal/l) See note 9.

Carbohydrate: Metabolisable carbohydrates shall provide at least 75 % of total energy. See note 10

Sodium: The sodium content shall be at least 20 mmol/l (460 mg/l) as sodium ions (Na^+) See note 11 and not greater than 50 mmol/l (1150 mg/l) as sodium ions (Na^+).

Note 7 – The SCF discusses carbohydrate-electrolyte solutions which are intended for rehydration. However there exists on the market today a range of drinks produced to satisfy particular needs associated with intense muscular activity. Interested parties have suggested that 3 electrolyte solutions should be included in the Directive:

a) Carbohydrate-electrolyte solutions: designed to provide optimal hydration and carbohydrate energy before, during and after the expenditure of intense muscular effort. It has been suggested that these products may provide energy between 80-350 kcal/l and contain 20-50 mmol sodium/litre.

b) Low calorie carbohydrate-electrolyte solution: designed to provide optimal hydration before, during an after the expenditure of intense muscular effort. It has been suggested that these products could provide a more limited amount of energy between 60-100 or 200 kcal/l and contain 20-50 mmol sodium/litre.

c) Electrolyte solutions: designed to provide fluid and electrolytes for thirst quenching, general hydration and help to top up the electrolytes lost in sweat, before, during and after the expenditure of intense muscular effort. It has been suggested that these could be energy free or have a minimum energy content of 60 kcal/l and contain 6.5-20 mmol sodium/litre.

Note 8 – As indicated in note 7, it has been suggested that the minimum energy content of drinks should be lower to permit specially adapted drinks to be produced which promote hydration but which are not calorific for use by individuals who are being physically active to help reduce their weight or for individuals who need to maintain a relatively low body weight. Certain interested parties have suggested that the electrolyte solutions might be energy free whilst others have proposed a minimum energy content of carbohydrate-electrolyte solutions of 60 kcal/l.

Note 9 – It has been suggested that the maximum energy content should be up to 1100 kcal/l as the maximum of 350 kcal/l is too low for recovery drinks formulated for both energy provision and hydration.

Note 10 –There are some carbohydrate-electrolyte solutions that contain peptides, amino acids, or triglycerides and may contain less than 75 % of energy from metabolisable carbohydrates.

Note 11 – The SCF report noted that carbohydrate electrolyte solutions should contain 460-1150 mg (20-50 mmol) sodium/l. Interested parties have indicated that there are products on the

Osmolality: The osmolality shall be at least 200 mOsml/kg water and not greater than 330 mOsml/kg water. See note 12

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market that are specially formulated for general hydration rather than optimal hydration and can help top up electrolytes lost in sweat. Such products have a sodium content of 150-460 mg (6.5-20 mmol) sodium/l which interested parties have indicated will help avoid hyponatraemia.

Note [12] – The Commission services has interpreted the SCF report comment on osmolalities of carbohydrate-electrolyte drinks as being an essential requirement. The wording of the SCF report is not clear on this point and it states that carbohydrate electrolyte solutions "...may be formulated to cover a range of osmolalities between 200 and 330 mOsml/kg water.". It is not clear if the wording means that the stated range of osmolalities is an essential requirement for carbohydrate-electrolyte drinks or is optional.

Interested parties have requested that the osmolality of electrolyte solutions should not be specified in the Directive.

PROTEIN CONCENTRATES

3. Products referred to in point (c) of Article 2 shall contain:

Protein content: At least 70 % of dry matter shall be protein.

Protein quality: The Net Protein Utilisation (NPU) should be at least 70 %. See note 13

If vitamin B_6 is added the product shall contain at least 0.02 mg vitamin B_6 per g protein.

The addition of amino acids is permitted solely for the purpose of improving the nutritional value of the proteins and only in the proportions necessary for that purpose. See note 14

Note 13 – In the SCF report on the revision of the essential composition of infant formulae and follow-on formulae it was proposed that protein quality should not be based on Net Protein Utilisation. In the report it is proposed that protein quality should be based on the chemical index or amino acid score of a protein in comparison with a reference protein.

Note 14 – Interested parties have suggested that addition of amino acids should also be permitted for reasons other than improving the nutritional value of protein, for example for erogenic and performance improving reasons. In addition, they have indicated that in certain circumstances when carbohydrates are in short supply amino acids may be used as a source of energy and that some amino acids are used more heavily so some deficiency may occur. It has been suggested that there should also be provision for the addition of dipeptides.

PROTEIN ENRICHED FOODS

4. Products referred to in point (d) of Article 2 shall contain:

Protein content: At least 25 % of total energy shall be from protein. See note 15

Protein quality: The Net Protein Utilisation (NPU) shall be at least 70 %. See note 13

If vitamin B_6 is added the product shall contain at least 0.02 mg vitamin B_6 per g protein.

The addition of amino acids is permitted solely for the purpose of improving the nutritional value of the proteins and only in the proportions necessary for that purpose. See note 14

Note 15 –The criteria of 25 % of energy from protein could mean that low calorie products that provide nutritionally insignificant amounts of protein could be described as a "protein enriched food".

Health Risks of Selected Performance-Enhancing Drugs

Peter A. Chyka, PharmD, DABAT, FAACT

This article reviews adverse effects of and the difficulty of attributing toxic effects to selected drugs and dietary supplements that purportedly enhance athletic performance. On surveys estimating the extent of performance-enhancing drug use, 5% of high school students indicated anabolic-adrenergic steroid use, and approximately 28% of collegiate athletes and 5.6% of middle and high school athletes admitted creatine use. Many adverse health effects from the abuse of androgenic-anabolic steroids and androstenedione (a prodrug) are exaggerations of excessive testosterone on hepatic, cardiovascular, reproductive, and behavioral functions that can produce permanent changes. With creatine use, nausea, vomiting, diarrhea, elevated serum transaminase concentrations, hypertension, fluid retention, muscle cramping, and muscle strains have been reported. Ephedra stimulates adrenergic receptors, leading to tachycardia and hypertension, with central nervous system effects of anxiety, tremor, and hyperactivity. From 1997 to 1999, 10 people died and 13 suffered permanent disabilities due to ephedra. γ-Hydroxybutyrate and several prodrugs (γ-butyrolactone and 1,4-butanediol) can produce alternating agitation and coma, amnesia, hypotonia, ataxia, nystagmus, tremors, bradycardia, respiratory depression, and apnea. Although γ-hydroxybutyrate abuse began as a bodybuilding aid, most serious adverse effects are from acute overdoses. Adverse effects from performance-enhancing drugs do occur, but their extent and frequency are unknown.

KEY WORDS: Androgenic-anabolic steroid toxicity, androstenedione toxicity, creatine toxicity, ephedra toxicity, γ-hydroxybutyrate toxicity, performance-enhancing drugs, health hazards.

> I'd asked a red-shirted clerk whether he warned kids away from certain supps. . . . "But do you tell them about side effects, or steer them to safer things?"
>
> —Paul Solotaroff ("Killer Bods," *Rolling Stone*, February 14, 2002)

A reporter from *Rolling Stone* magazine captured an essential element of the hazards and abuse of performance-enhancing drugs—sales clerks, friends, coaches, and other athletes are the perceived experts on the use and risks of these agents. The risks and health hazards of performance-enhancing drugs are difficult to characterize for several reasons.[1,2] A prime reason is that many of the substances used by athletes are illegal or banned by sports organizations. Admitting to their use is counterproductive to an aspiring athlete and thereby compromises the reliability of surveys of usage and adverse effects. Another reason is that many people who abuse performance-enhancing drugs will seek medical help only when serious side effects occur or disagreeable effects, such as hirsutism or gynecomastia, develop. Many individuals use a variety of agents with varying regimens, thereby making it impossible to link a given agent with an adverse effect reliably. Because of ethical concerns, clinical trials are impossible to conduct with the doses and regimens typically used by athletes. Long-term studies are similarly difficult to conduct. The remaining evidence includes a collection of case reports and anecdotal reports of overt abuse or acute overdose. Some reports point clearly to the cause, while most are clouded by the sequential use of multiple drugs; the combined used of several agents; complications of underlying conditions; the use of specialized diets; and the influence of multiple risk-taking behaviors, such as abusing other drugs, injecting drugs, and engaging in unprotected sexual intercourse with multiple partners. Many

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of the substances used to enhance athletic performance are clandestinely manufactured, which introduces the additional risk of adulterants and contaminants to further complicate assignments of toxicity. For athletes, coaches, parents, trainers, merchants, and health care professionals, there is little or no incentive to report adverse effects from performance-enhancing drugs. In this setting, a cause-and-effect relationship is difficult to establish.

To further obscure the issue, in the United States, there are 3 categories of products that are regulated for human consumption: foods, drugs, and dietary supplements. The establishment of the category of dietary supplements by Congress in 1994 through the Dietary Supplement Health and Education Act distinguished these agents from drugs in several notable ways.[3-5] Dietary supplements are not subject to premarketing review and approval by the US Food and Drug Administration (FDA) to establish safety, efficacy, and purity. Further, the burden to demonstrate a dietary supplement as unsafe is shifted to the FDA because manufacturers of dietary supplements cannot be compelled to undertake postmarketing surveillance.[5,6] With the widespread use of dietary supplements for a variety of conditions, including the enhancement of athletic performance, the potential for adverse effects is great, although it is difficult to monitor and assess the true risk.

The purpose of this article is to review the adverse effects associated with performance-enhancing drugs, such as androgenic-anabolic steroids, and several dietary supplements, such as androstenedione, creatine, ephedra, and γ-hydroxybutyrate (GHB). Health care professionals should recognize the potential risks of these agents, provide appropriate advice, and appreciate the problems in determining their adverse effect profiles.

ANDROGENIC-ANABOLIC STEROIDS

Androgenic-anabolic steroid use by athletes and nonathletes has been prevalent since the 1950s to enhance athletic performance, increase muscle size and/or reduce body fat, or engage in a pattern of high-risk behaviors by adolescents.[1,7] More than 40 different androgenic-anabolic steroids are available (Table 1) and are chemical variations of testosterone. Androgenic-anabolic steroids are taken orally; injected intramuscularly; or applied topically as creams, gels, or drug delivery patches. Typically, the 17-α-alkylated androgenic-anabolic steroids are taken orally and have been associated with an increased risk for liver injury.[2] Androstenedione is an androgen produced by the gonads and adrenal gland and is a major precursor to testosterone. When androstenedione is consumed as a dietary supplement, it can increase serum testosterone and estradiol in men.[8] Testosterone and the other androgenic-anabolic steroids circulate in the bloodstream and interact with androgen receptors in tissues throughout the body to produce androgenic (virilizing) and anabolic (muscle-promoting) effects.[2,9] A small proportion of testosterone is also metabolized to estradiol, which can lead to feminizing effects when taken in excess (Figure 1). Although these drugs are classified as Schedule III controlled substances in the United States, they can be illegally and readily obtained by diversion from veterinary sources, smuggling from other countries such as Mexico, theft from pharmacies, or manufacture by clandestine laboratories.[1,9] All androgenic-anabolic steroids are banned for use by athletes who wish to compete in the Olympics, but some professional sports organizations, such as Major League Baseball, do not monitor for the use of androgenic-anabolic steroids. A drug testing program for Major League Baseball is being considered for the 2003 season.

Table 1
Examples of Commonly Used Anabolic-Androgenic Steroids

Oral agents
Methandrostenolone (Dianabol)
Methyltestosterone
Oxandrolone (Oxandrin)
Oxymetholone (Anadrol)
Stanozolol (Winstrol)
Parenteral agents
Boldensone undecylenate (Equipose)
Nandrolone decanoate (Deca-durabolin)
Nandrolone phenproprionate (Durabolin)
Testosterone cypionate (Depo-testosterone)

> "Yeah, I won't sell 'em andro," he whispered, "especially if they're young, although their parents'll buy it for 'em anyway." (Paul Solotaroff, "Killer Bods," *Rolling Stone*, February 14, 2002)

The types of androgenic-anabolic steroids used and their patterns of use vary by sport, locale, and availability. Typical doses of androgenic-anabolic steroids used to enhance athletic performance exceed therapeutic doses by 10-fold to 100-fold.[1,2,9] During a cycle, abusers can reach 40 to 100 times the daily dose used therapeutically for testosterone (6 to 10 mg) to treat hypogonad-

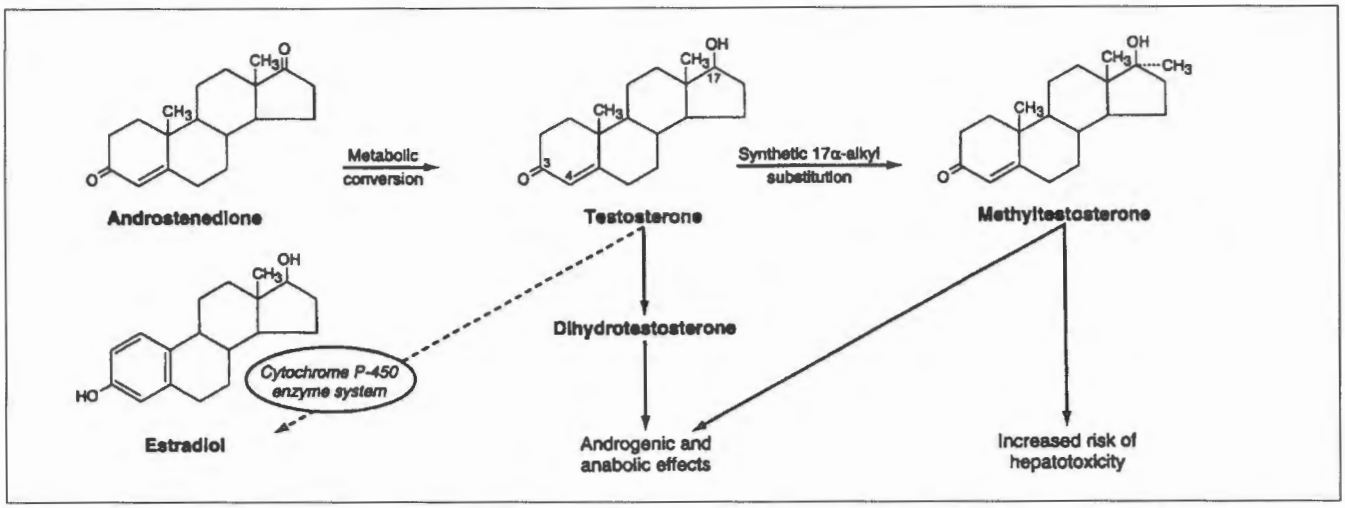

Figure 1. *Relationship of testosterone, precursors, synthetic analogs, and metabolites to effects. Source: Reprinted from reference 9 (Figure 72-2, p 597) and used with permission of W. B. Saunders Company.*

ism in male patients.[10] Many athletes use regimens to "stack" their drug use by taking one or more different androgenic-anabolic steroids and occasionally mixing dosage forms, with the belief that the interaction of androgenic-anabolic steroids will enhance the muscle-building effects. Another common practice involves a "pyramid" regimen, whereby androgenic-anabolic steroids are taken in cycles of increasing and decreasing doses over 4 to 18 weeks, with occasional drug-free periods.[10] The intent is to allow time for the body to adjust to the high doses of androgenic-anabolic steroids while restoring normal hormonal balance during the drug-free period. Neither of these approaches has been proved safe or effective.[1] Often, other drugs are taken during androgenic-anabolic steroid abuse to minimize potential adverse effects.[2] To reduce the estrogenic effects of high-dose androgenic-anabolic steroids use, such as increased high-pitched voice and gynecomastia, male users will self-administer the antiestrogenic drug tamoxifen.[9,11,12] Others will take chorionic gonadotropin to stimulate testicular testosterone production to reduce the extent of testicular atrophy and its adverse effects on sperm production and motility induced by androgenic-anabolic steroids.[9,11,13]

Many of the adverse health effects of androgenic-anabolic steroid abuse typically are exaggerations of the physiological effects of testosterone from excessive doses or chronic exposure (Table 2). Generally, higher doses of anabolic-adrenergic steroids for prolonged periods will increase the likelihood of exaggerated hormonal effects. A 1984 position stand by the American College of Sports Medicine on the use of anabolic-adrenergic steroids in sports highlighted 4 effects of major concern: adverse effects on the hepatic, cardiovascular, reproductive, and behavioral functions.[14]

Elevated liver function tests (aspartate aminotransferase, alanine aminotransferase, bilirubin, lactic dehydrogenase, alkaline phosphatase) have been associated with the abuse of androgenic-anabolic steroids, particularly the 17-α-alkylated steroids. These elevations typically return to baseline values after discontinuing use. Dose-dependent jaundice and hepatic dysfunction are likely to become manifest after 2 to 5 months of supraphysiological doses.[2] The abuse of androgenic-anabolic steroids has also been associated with peliosis hepatis (blood-filled hepatic cysts that can rupture) and liver tumors. The relationship to androgenic-anabolic steroid use is circumstantial or coincidental. Most tumors are benign but are more likely with the 17-α-alkylated steroids.[14]

The cardiovascular system is affected acutely by elevated blood pressure secondary to blood volume increases and fluid retention.[2] Androgenic-anabolic steroid use has been associated with decreased high-density lipoprotein, increased low-density lipoprotein, and altered glucose tolerance.[14] These effects may increase the potential for thrombus formation, but a relationship has not been established. Similarly, left ventricular hypertrophy has been observed, but its causation by androgenic-anabolic steroids is unclear.[14]

The reproductive and hormonal effects of androgenic-anabolic steroids are clearly associated with the use of supraphysiological doses.[9,14] In men,

Table 2
Possible Adverse Effects of Anabolic-Androgenic Steroid Abuse

Hormonal system
Men
Infertility
Breast development
Testicular atrophy
High-pitched voice
Women
Enlargement of the clitoris
Excessive growth of body hair
Low-pitched voice
Both sexes
Male pattern baldness
Musculoskeletal system
Short stature with childhood use
Tendon rupture
Cardiovascular system
Myocardial infarction
Left ventricular hypertrophy
Liver
Elevated liver function tests
Peliosis hepatis
Tumors
Skin
Acne and cysts
Oily scalp
Infection
HIV/AIDS
Hepatitis
Psychiatric effects
Rage and mania
Delusions

Source: Adapted from reference 1.

androgenic-anabolic steroid use leads to oligospermia, azoospermia, and testicular atrophy. Because of the metabolism of some testosterone to estradiol, gynecomastia and a higher pitched voice are possible. In women, the use of androgenic-anabolic steroids produces enlargement of the clitoris, hirsutism, deepening of the voice, and amenorrhea, which may persist after discontinuation of androgenic-anabolic steroid use. Both sexes can develop male pattern baldness, disproportionate development of the upper torso, acne, sebaceous cysts, and an excessively oily scalp.[9,14] The rapid and excessive development of muscles can promote tendon rupture.[1] Children who use androgenic-anabolic steroids may prematurely stunt their growth by the closure of the epiphyseal plates of their bones, develop precocious puberty (in boys), and exhibit contrasexual precocity (in girls).[9]

The behavioral effects of androgenic-anabolic steroids have been noted in both sexes to include increased or decreased libido, mood swings, and aggressive behavior.[14] The phenomenon of "steroid rage" is loosely characterized as aggressive, violent behavior and may be associated with preexisting psychiatric illness that is exacerbated by the use of androgenic-anabolic steroids or other substances or may be related to an addiction to these agents.[2] The extent to which the abuse of androgenic-anabolic steroids contributes to violence and behavioral disorders is unknown.

For individuals who inject androgenic-anabolic steroids, the additional risks for local and systemic infections from bacteria, HIV, hepatitis B and C, and fungus from contaminated needles or unsterile injection techniques are present.[1,9] Since some androgenic-anabolic steroids are manufactured illegally in unsterile and uncontrolled conditions, there is also a risk for contaminated and adulterated products.

The long-term health risks to athletes who use androgenic-anabolic steroids are difficult to study. There may be a higher occurrence of cardiovascular disease, mental illness, and neoplasms in athletes who have used androgenic-anabolic steroids compared to those who have not.[15] There has also been an association of sudden death in users of androgenic-anabolic steroids, but the evidence is unconvincing to date.[15] These potential consequences must be considered in the perspective of the other practices of athletes, such as unusual diets, the use of other drugs and supplements, risk-taking behaviors, extreme conditioning, and underlying disease such as an unrecognized cardiovascular congenital anomaly, that may influence the outcome.

A recent review of the risk factors associated with androgenic-anabolic steroid use in adolescents reveals that users are more likely to be boys; to use other illicit drugs, alcohol, and tobacco; and to be athletes engaged in football, wrestling, weight lifting, and bodybuilding.[16] The use of androgenic-anabolic steroids may begin as early as the middle school years. An estimated 16% to 36% of adolescents who use androgenic-anabolic steroids do not participate in sports and abuse these drugs to change their appearance because of dissatisfaction with their body image.[16] Several behaviors and characteristic changes may indicate the use of androgenic-anabolic steroids in adolescents and may serve to identify those who are potentially at risk for adverse effects (Table 3).[17(pp78-79)]

There is some association among adolescents, particularly boys, who use anabolic-adrenergic steroids and engage in other high-risk behaviors, such as driving after drinking alcohol, carrying a gun, not using a condom during intercourse, and suffering injury from a physical fight.[7] A survey of youth risk behaviors among US high school students during 2001 found that 5% of

Table 3
Characteristics Associated With Abusers of Androgenic-Anabolic Steroids

Recent personality or behavioral changes
Strength or power athlete, weight lifter
Recent significant increase in appetite and food consumption
Rapid weight gain of 25 pounds or more over 3 to 6 months
Recent gains in muscular strength and build
Use of other supplements for muscle building
Use of other drugs to self-treat steroid-related adverse effects, for example, tamoxifen, acne medications, diuretics, anxiolytics
Frequent posing in front of mirrors
Recent increase in time spent in the weight lifting gym
Reads or subscribes to "muscle magazines"
Frequently attends or competes in bodybuilding contests
Employed as a nightclub "bouncer," professional wrestler, male nude dancer, or law enforcement officer and is overly muscular
Claims to have stopped using androgenic-anabolic steroids
Has an older brother who is a strength or power athlete

Source: From *Macho Medicine: A History of the Anabolic Steroid Epidemic*, pp 78-79. © 1991 William N. Taylor by permission of McFarland & Company, Inc., Box 611, Jefferson, NC 28640. www.mcfarlandpub.com

students had used illegal anabolic-adrenergic steroids during their lifetimes, with use more likely among boys (6.0%) than girls (3.9%) and among white students (5.3%) than black students (3.2%).[18]

CREATINE

Creatine is an amino acid derivative that is synthesized in the liver, kidneys, and pancreas and supplied by the diet primarily in meat and fish. Cells with high energy requirements, such as muscle cells, use creatine in its phosphylated form, phosphocreatine, to generate adenosine triphosphate (ATP) from adenosine diphosphate (ADP). Oral supplementation with creatine monohydrate is thought to increase the pool of available phosphocreatine to enhance anaerobic adenosine triphosphate production and promote the resynthesis of phosphocreatine during the aerobic recovery phase after exercise.[19] Creatine use is widespread among adults and adolescents, with approximately 28% of collegiate athletes and 5.6% of middle and high school athletes admitting creatine use.[20]

Creatine supplementation causes the retention of creatine in tissues and a reduction in urine production, resulting in muscle enlargement due to water absorption and rapid-onset weight gain due to water retention.[21] Renal dysfunction due to creatine use has been demonstrated in animals,[22] but only one case of interstitial nephritis that improved with the discontinuation of creatine has been reported in a patient with preexisting glomerulosclerosis.[23] The link of adverse effects of creatine on the kidneys is unproved in humans.[21,22] Patients with preexisting kidney disease, diabetes mellitus, who are pregnant, or who are taking potentially nephrotoxic drugs should avoid creatine supplementation until safety is established.[19,21]

> "Look, all that stuff is legal. If it was dangerous, they couldn't put it out there." (Paul Solotaroff, "Killer Bods," *Rolling Stone*, February 14, 2002)

Twenty reports of adverse reactions from creatine had been voluntarily filed with the FDA by 1998, but causality was inconclusive.[19] Creatine was suspected as a contributing factor in the deaths of 3 college wrestlers who experienced severe dehydration and heat intolerance, but the claim could not be clearly substantiated, and the more likely cause was a rapid weight loss program in hot conditions with fluid restriction.[24] Anecdotal reports of nausea, vomiting, and diarrhea have been reported, as well as cases of elevated serum transaminase concentrations, hypertension, muscle cramping, and muscle strains.[21] Muscle dysfunction has not been observed.

After a review of the evidence of adverse effects from creatine supplementation, Poortmans and Francaux[25] concluded that "there is no evidence for deleterious effects in healthy individuals," while an expert panel of the American College of Sports Medicine[21] concluded that "the lack of adverse effects does not equal safety." More studies of humans taking regimens employed by athletes may provide a better estimation of the potential risks.

EPHEDRA

Various Asiatic species of the evergreen *Ephedra* contain ephedra alkaloids composed of ephedrine, pseudoephedrine, and other ephedrine derivatives.[26]

The ephedra alkaloid–containing species are collectively known as *ma huang*. Numerous ephedra-containing products are available on the market and are primarily promoted as weight loss products and athletic performance-enhancing agents. Common products that contain ephedra with other agents include Metabolife356 and Ripped Fuel; ephedra is also referred to as "herbal ecstasy." Ephedra-containing products are widely available at nutrition stores, pharmacies, convenience stores, and through the Internet. Products containing ephedra are classified as dietary supplements and are not subject to the same FDA guidelines for the establishment of safety, purity, and effectiveness that apply to drugs.[6] There is considerable variability in content among products, as evidenced by a study of 20 products that found that ephedra content ranged from 0% to 154% of the amount claimed on the label, with considerable lot-to-lot inconsistency.[27]

Ephedra produces amphetamine-like actions that stimulate α-adrenergic and β-adrenergic receptors and promote the release of norepinephrine from nerve endings. These actions primarily increase heart rate and peripheral vascular resistance, leading to hypertension.[26] Effects on the central nervous system include anxiety, tremor, and hyperactivity.

> "And with ephedrine, I try to offer a stimulant-free product . . . ." "Ephedrine is evil . . . how can they even sell this stuff?" (Paul Solotaroff, "Killer Bods," *Rolling Stone*, February 14, 2002)

An in-depth, structured review of the adverse events reported for ephedra alkaloids to the FDA from 1997 to 1999 revealed 140 reports.[28] Adverse effects were definitely or probably related to the use of ephedra in 31% of the cases, and 31% were possibly related. The effects noted in the reports included hypertension (17 reports); palpitations, tachycardia, or both (13 reports); stroke (10 reports); and seizures (7 reports). Ten people died and 13 individuals suffered permanent disability. Many of the cases involved products that contained caffeine, and the authors argued that the interaction of caffeine and ephedra produces an additive stimulant effect on the cardiovascular and nervous systems, thereby increasing the risk for serious events. A similar review conducted on FDA reports submitted from 1995 to 1997 identified 926 cases of possible ma huang toxicity.[29] In 37 patients, the use of ma huang was associated with adverse events such as stroke (16 reports), myocardial infarction (10 reports), and sudden death (11 reports).

The evidence linking ephedra to adverse effects in both of these studies[28,29] is circumstantial and relies on voluntary reporting to the FDA. However, the events described by these reports are consistent with toxicity reported for ephedrine.[26,29] The adverse effects of ma huang have been linked with the concurrent use of caffeine (commonly found in supplements as guarana) and exercise.[30] Whether the effects of ephedra alone, in combination with other stimulants, underlying cardiovascular disease, or a combination of these factors is responsible for the serious adverse effects of ephedra remains unresolved.[6] The prevalence of the use of nonprescription weight loss products in the United States from 1996 to 1998 was estimated to be 7% of the adult population, with 1% claiming the use of ephedra products.[31] The extent of ephedra use during athletic performance or conditioning is unknown. Ephedra is a banned substance in the Olympics.

GHB AND PRECURSORS

GHB (γ-hydroxybutyrate) has been promoted to bodybuilders since the 1980s as an adjuvant to growth hormone, which would increase muscle development, burn fat, produce euphoria, and improve sleep. In the 1990s, GHB, purported to be an aphrodisiac, became popular at all-night dance parties and has been used as a "date rape" drug with names such as Liquid Ecstasy, Liquid X, and Organic Quaalude. It is one of several drugs known as "club drugs," which also include methylenedioxy-methamphetamine, flunitrazepam, and ketamine, which are used to enhance the social experience at events such as all-night dance parties known as "raves."[32,33] In March 2000, GHB became a Schedule I controlled substance because of increasing cases of toxic reactions and deaths associated with its use. With the FDA restricting the sale of GHB, which could previously be purchased at nutrition stores and over the Internet, a number of prodrugs surfaced, such as γ-butyrolactone (GBL) and 1,4-butanediol (1,4-BD), which is an industrial solvent. These prodrugs can be converted to GHB in clandestine laboratories with simple reactants or endogenously by existing metabolic pathways (Figure 2).[34,35] Restrictions on the sale of GBL and 1,4-BD have led to hoarding of the chemicals and posting of recipes on the Internet.[32,34-36]

GHB is naturally found in the central nervous system and is thought to regulate a number of homeostatic functions such as sleep, body temperature, cerebral glucose metabolism, memory, and emotional control. Its pharmacological effects appear to stem from its influence on dopaminergic activity through GHB or γ-amino-

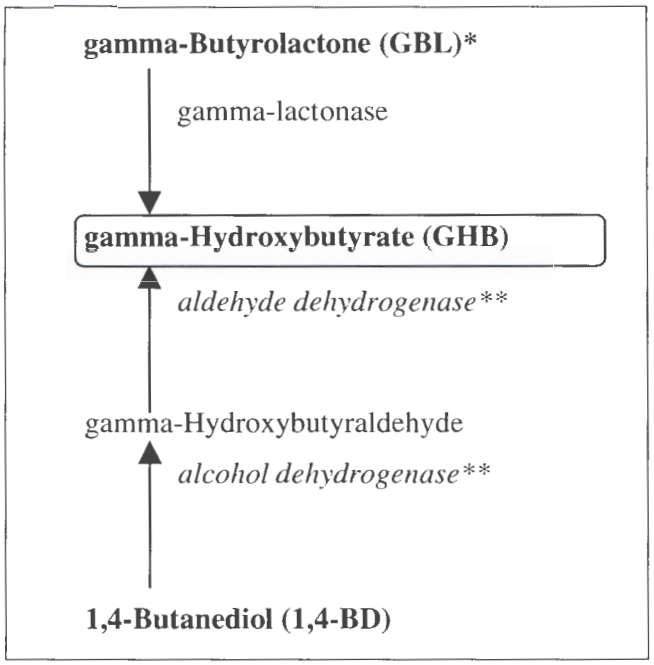

Figure 2. *Metabolic conversion of exogenous precursors to γ-hydroxybutyrate.*
*Reaction with sodium hydroxide prior to ingestion can produce GHB.
**The coingestion of ethanol may lead to competitive metabolism and prolonged toxicity.

butyric acid receptors.[34] In July 2002, the FDA approved GHB, also known as sodium oxybate (Xyrem by Orphan Medical), for the treatment of narcolepsy as a Schedule III drug with special restrictions on its dispensing.[37] For illicit uses, GHB and GBL will remain Schedule I drugs.[38]

The primary neurotoxic effects of GHB and related agents on overdose include alternating agitation and coma, amnesia, hypotonia, ataxia, nystagmus, and tremors. Other toxic effects include bradycardia that reverts to tachycardia on recovery, respiratory depression and apnea, and nausea and vomiting, which are predispositions to aspiration pneumonitis. Sometimes, flushing, hypothermia, and diaphoresis are present. Most symptoms resolve within 2 to 8 hours, but deaths have been reported.[32,34-36] A GHB withdrawal syndrome characterized by anxiety, insomnia, and tremor may develop soon after the discontinuation of chronic GHB use.[39] Although GHB abuse apparently began as an aid to bodybuilding, most of the reports of serious adverse effects are from acute overdoses.

CONCLUSION

Adverse effects from performance-enhancing drugs do occur, but the extent and frequency of these reactions are unknown. Although adults can suffer permanent changes from some agents, children are most vulnerable to the toxic effects on their developing bodies. There are other chemicals that pose risks to athletes, such as ethanol,[40] caffeine,[41] cocaine,[42] and erythropoietin,[43] and their exclusion here does not imply benign effects. Measures to control the abuse of these agents through routine monitoring in competitive sports is noteworthy, and education on the risks of these agents may be a potential deterrent. As the vignettes from *Rolling Stone* attest, the problem is multifactorial. To help stem the abuse of a group of drugs that are clearly dangerous, anabolic-androgenic steroids and club drugs, the National Institute on Drug Abuse has created informational Web sites at http://www.steroidabuse.org and http://www.clubdrugs.org. Other widespread educational programs are indicated to educate athletes, coaches, parents, and health care professionals on the known health risks of these agents.

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